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Moisture-stress induced sterility and outcrossing in spring wheat (*Triticum aestivum* L.)

by

Oliver Kipchoge Kiplagat ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Plant Breeding

Department of Plant Science

Edmonton, Alberta

Fall, 1995

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Moisture-stress induced sterility and outcrossing in spring wheat (*Triticum aestivum* L.)** submitted by **Oliver Kipchoge Kiplagat** in partial fulfillment of the degree of Master of Science in Plant Breeding.

KG Briggs

Keith G. Briggs (Supervisor)

Anne M. Johnson-Flanagan (Supervisor)

Francis Yeh (Member)

To Angela Jepkoech,

Always remembered.

ABSTRACT

Pre-anthesis moisture stress increased the frequency of sterile florets in semidwarf and tall wheat cultivars. The increase in floret sterility resulting from moisture stress in the semidwarf cultivars was higher than in the tall cultivars. Hand pollination of moisture stressed plants of Cutler (a semidwarf cultivar), and Roblin (a tall cultivar) increased the seed set from 21 to 62% in Cutler and from 60 to 72% in Roblin. This indicates that moisture stressed Cutler had a higher frequency of male-sterile florets compared with stressed Roblin. Moisture stress also increased the extent of floret opening in the two cultivars, resulting in increased potential of the florets to receive external pollen. Exposure of moisture stressed plants to pollen from a marker stock increased the seed set from 9 to 48% in Cutler, and from 42 to 49% in Roblin, suggesting that there was more outcrossing in Cutler than in Roblin. Analysis of seed from moisture stressed plants that were exposed to pollen from the marker stock by phenotypic observation of the F_1 plants, seed protein electrophoresis of the F_1 seed and RAPD analysis of DNA from F_1 plants, all indicated that there was more outcrossing in Cutler than in Roblin.

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CHAPTER 1

LITERATURE REVIEW

1. 1 SEMIDWARF AND TALL WHEATS

Traditionally, cultivated wheat (*Triticum aestivum* L.) varieties were tall and reached heights of around 150-170 cm (Law et al, 1978). Under modern cultivation in which high levels of artificial fertilizer are in common use, such wheats perform poorly mainly because their height makes them very susceptible to lodging. Despite strong selection, progress in breeding for reduced height was initially very slow because plant height in wheat is a character controlled by many genes, with most of the 21 chromosomes of hexaploid wheats carrying genes affecting it (Kuspira and Unrau, 1957; Law and Worland, 1973b).

The breakthrough in height reduction in wheat was realized with the discovery of major dwarfing genes in the Japanese variety 'Norin 10'. Use of the Norin 10 dwarfing genes in breeding programs, particularly by the International Centre for Maize and Wheat Improvement (CIMMYT) resulted in high yielding semidwarf wheats which were in part responsible for the 'Green Revolution', especially in the low altitude countries of the world (Lupton, 1987). These height reducing (Rht) genes have been mapped to chromosomes 4A and 4D in wheat (Gale and Marshall, 1976; Gale et al, 1975). Apart from imparting lodging resistance, the dwarfing genes have been reported to increase the harvest index, and the number of seed bearing tillers (Jain and Kulshrestha, 1976).

Several researchers have reported that compared with tall types, semidwarf wheat cultivars are more productive under favourable moisture conditions (Syme, 1969; Laing and Fisher, 1977; Pearman et al, 1978; Allan, 1986). However, they have also been reported to be more susceptible to drought and high temperature stress (McNeal et al, 1972; Laing and Fisher, 1977; Woodruff and Tongs, 1983).

1.2 CANADA WESTERN RED SPRING AND CANADA PRAIRIE SPRING WHEAT CLASSES

More than 90% of Canadian wheat is produced in the prairie provinces of Manitoba, Saskatchewan and Alberta (Anon., 1994). This is a region with long, cold winters, and hot, short summers. There is usually limiting rainfall, and droughts are common. Although the relatively low rainfall limits yield, it is also an important factor in producing grain with high protein and high baking quality. Several classes of spring wheat are produced in western Canada. These include the Canada Western Red Spring wheat (CWRS), which has mainly tall cultivars, and the Canada Prairie Spring wheat (CPS), with mainly semidwarf cultivars. Most of the wheat grown in the Canadian prairies is of the CWRS type. The CWRS class is characterized by excellent baking and milling quality, adequate disease resistance and relatively good agronomic characteristics (Kibite, 1986). Despite their several desirable characteristics, CWRS cultivars have a disadvantage in that they are relatively lower yielding, compared to cultivars in the CPS class.

The need for alternative higher yielding wheat cultivars for the Canadian prairies led to the development of Canada Utility wheats and CPS wheat (Briggs, 1985; Kibite, 1986; Wolfe and Clarke, 1985). The semidwarf CPS wheats are characterized by good milling quality and adequate disease resistance, but are inferior to CWRS in protein content and baking quality. They have been reported to outyield CWRS cultivars by between 25 and 35 % (Wolfe and Clarke, 1985).

1.3 OFFTYPE OCCURENCE IN WHEAT FIELDS

As in most crops, plant to plant genetic and phenotypic uniformity in the field is a desirable characteristic in a wheat cultivar. In particular there have been concerns regarding the appearance of tall offtype plants in fields of many semidwarf cultivars. It is not known whether there is a higher frequency of offtypes in semidwarf fields as compared to fields of conventional height cultivars, or simply that the height difference makes it easier to observe offtypes in semidwarf wheats. Nonetheless, the apparent occurrence of significantly more offtypes in semidwarf cultivars has created problems for breeders and seed growers during the maintenance and increase of pedigreed seed. Offtype appearance in wheat fields may reduce grower acceptance of a variety and also presents problems during seed certification. Offtypes may also down-grade a crop and lower the economic value. This is particularly the case for seed crop production where strict regulations are followed to ensure the production of seeds that meet minimum purity standards. According to the FAO Quality Declared System of seed production, for example, at least 98% of the plants in a wheat field must conform to the morphological characteristics of the variety (Anon., 1993). In Canada the seed production rules permit a maximum of one offtype plant per 10,000 plants of CPS wheat in fields of Breeders' Foundation and Registered seed while a maximum of five offtype plants are allowed per 10,000 plants in fields of Certified seed (Anon., 1994a).

There are several different reasons for the occurrence of offtype plants in wheat fields. The main reasons include accidental mixing of seeds, volunteer plants, genetic instability and abnormalities, and outcrossing in previous generations. With careful seed handling, and rigorous cleaning of equipment and seed containers, mixing is not usually a problem. Proper crop rotation minimizes the likelihood of contamination from volunteer plants. Offtypes resulting from mixing, or volunteer plants, can also be eliminated through routine procedures like rogueing.

Most of the reported cases of offtypes in wheat cultivars involve tall plants appearing in fields of semidwarf cultivars (Worland and Law, 1985; Storlie and Talbert, 1993). Tall, awnless plants are often observed in fields of CPS semidwarf cultivars such as Cutler (Briggs, Pers. comm.). Offtypes in some seed stocks of Cutler have reappeared after rogueing, suggesting that segregation for offtype plants was still occurring. This also implies that these offtypes did not result from seed mixing or volunteer plants.

1.3.1. GENETIC ABNORMALITIES

Common wheat is a hexaploid (2n=6x=42) with a chromosome number of 42. It has three genomes, designated A, B and D. Hexaploid wheats are believed to have evolved from a prototype diploid species that underwent a divergent evolution to give three progenitor diploid species, which later converged to give the hexaploids (Riley, 1975). Through cytogenetic studies, it has been established that common wheat contains the full set of chromosomes from *Triticum turgidum* (genome AABB). This has led to the conclusion that common wheat evolved from the hybridization of *T. turgidum* with *T. tauschii*, which contributed the D genome (McFadden and Sears, 1946). Wheat is able to tolerate some genetic abnormalities such as aneuploidy because it contains three closely related genomes (Riley, 1975). Aneuploids may occur as a result of bivalent pairing failure during metaphase I of meiosis. Monosomics (2n-1) were found to occur with a frequency of about 0.19% in five euploid cultivars (Riley and Kimber, 1961).

Monosomy has been reported as a possible cause of offtypes in wheat. Worland and Law (1985) suggested that some tall offtypes in fields of a semidwarf wheat resulted from a monosomic condition at the 4D locus, which is also the locus of a gibberellin insensitivity gene responsible for height reduction (Gale et al., 1975; Gale and Marshall, 1976). Storlie and Talbert (1993) also reported that some tall offtype plants in a semidwarf spring wheat were a result of a monosomic 4B condition. These offtypes, which occurred spontaneously with a frequency of 0.2%, showed electrophoretic bands of glutenins similar to those of the pure cultivar, suggesting that they were not outcrosses. Rogueing of such offtypes could never eliminate these tall offtypes, as new ones would reoccur.

1.3.2 OUTCROSSING

Outcrossing is another factor that could lead to offtypes in wheat fields. In most crops, a lot of care is taken during seed production to minimize chances of pollen contamination that could lead to outcrossing. Seed production fields, especially for cross pollinated crops, are adequately isolated from neighbouring fields of the same crop. In rye and maize, for example, the minimum isolation distances for fields of pedigree seed is 300 m (Anon., 1994). In contrast, little attempt is made to prevent outcrossing in wheat fields since it is believed that as a self pollinated crop the chances of cross pollination are minimal. The FAO wheat seed production guidelines, for example, state that the seed field 'shall be isolated from all other fields of wheat or crop species with similar seed size by a distance adequate to prevent mechanical mixture, or by a physical barrier like a fence' (Anon., 1993). Similarly, the Canadian seed production regulations require an isolation distance of only a metre for wheat. However, the fact that seed set levels as high as 75% have been achieved in male sterile wheat lines during hybrid seed production (Virmani and Edwards, 1984) indicates that certain plant and environmental factors can result in high levels of cross pollination in wheat.

1.4. FACTORS AFFECTING CROSS POLLINATION IN WHEAT

Wheat is normally considered a self pollinating crop because of its floral structure and flowering behaviour (Percival, 1921; Leighty and Sando 1924). The extent of natural outcrossing in cultivated varieties has been reported to range from 0-4% (Heyne and Smith, 1967). However, in hybrid seed production systems, male sterile lines commonly achieve seed sets as high as 75% from cross pollination. Variability in the extent of natural outcrossing in wheat can be attributed to variations in flowering behaviour, floral characteristics of the varieties, and to variation in environmental factors (Virmani and Edwards, 1984).

1.4.1. FLORAL CHARACTERISTICS AND FLOWERING BEHAVIOUR

The wheat inflorescence is a spike with a main axis (rachis) bearing spikelets separated by short internodes (Lersten, 1987). Each spikelet is a condensed reproductive shoot consisting of two subtending sterile bracts (glumes) usually enclosing up to five florets. The florets are attached alternately to opposite sides of the rachis, and one or more of the upper florets are usually sterile, resulting in only two or three kernels per spikelet (Leonard and Martin, 1963). Each floret is enclosed by two bract-like structures, the lemma and palea, and has three stamens and a pistil bearing two styles with feathery stigma branches. In common wheat, the lemmas could be awned or awnless. Wheat stamens are smaller and produce fewer pollen grains than other cereal grasses. Only about 450,000 pollen grains are produced per plant as compared to about 4 million for rye (*Secale cereale*) and 18 million for maize (*Zea mays* L.) (DeVries, 1971). Since the florets are enclosed by the glumes, wheat is essentially a self pollinating crop. Florets are usually closed prior to, and immediately following pollination.

Little work has been done recently to study the outcrossing potential of self pollinated cultivars in wheat since it is considered a self pollinating crop. Most of the studies relating to cross pollination in wheat were done with respect to hybrid seed production, where potential parents have usually been selected for cross-pollinating characteristics.

The plant factors that influence the extent of cross pollination in wheat include the opening of the glumes, the awnedness of the lemma, the size of the stigma, the duration of stigma receptivity and the exsertion of the stigma. Others are the number of pollen grains per anther and anther dimensions, the elongation of the filament, the extrusion of the anthers, anther dehiscence, the extent of pollen shed inside the floret, the longevity of pollen grain viability, and the duration of flowering (DeVries, 1971; Virmani and Edwards, 1984). Significant varietal differences have been observed in most of these floral attributes (Cahn, 1925; Kherde et al, 1967; DeVries, 1974a).

Differences in pollen receiving capacity as well as in pollination capacity have been reported (Joppa et al, 1968). Floret opening is a prerequisite for cross pollination. During flowering, the glumes of each spikelet open slightly and then close following pollination. Delay or failure in pollination prolongs flowering and, consequently, male sterile plants have a longer period of floret opening than fertile plants (Saran et al, 1971). Floret opening is controlled by two lodicules that are located at the base of the ovary (Percival, 1921; Kadam, 1933). At anthesis, lodicules increase in turgidity, pushing apart the lemma and palea and consequently aid in anther extrusion and pollen interception. Lodicules were found to increase in size until the sixth day after complete ear emergence (McNeal and Ziegler, 1975). It was also observed that lodicules from both emasculated and male sterile spikes were significantly heavier, wider, and thicker than those from male fertile spikes.

The duration of stigma receptivity is critical in determining the potential for outcrossing in wheat. Stigma receptivity has generally been measured indirectly in wheat by determining seed set on male sterile plants during periods of delayed pollination. Several studies have shown that stigma receptivity is dependent on both genetic and environmental effects (Bardier, 1960; Khan et al, 1973).

Outcrossing is also a function of the pollen mass in the air which, in turn, is a function of the amount of pollen produced per anther, extent of anther extrusion, the number of anthers per unit area, and plant height. DeVries, (1972) reported that pollen concentration 20 cm above the spike was considerably less than that at the spike level, which in turn was less than that measured 20 cm below the spike level. Joppa et al (1968) reported that the extent of anther extrusion had the largest direct effect on pollen shedding.

1.4.2. ENVIRONMENTAL FACTORS

The most important environmental factors influencing outcrossing in wheat are pollinator distance, wind speed and direction, and relative humidity. Numerous studies have been conducted on the effect of pollinator distance and wind direction on the seed set of male sterile wheat lines (Bitzer and Patterson, 1967; Rajki and Rajki, 1968; Anand and Beri, 1971; DeVries, 1974b; Miller and Lucken, 1976). These studies indicate that seed set reduces with pollinator distance. In an experiment where pollinator distance ranged between 1.5 and 7.6 m, Bitzer and Patterson (1967) observed that wind direction affected seed set on male-sterile wheat lines as much as the pollinator distance.

Johnson and Schmidt (1968) found that pollen could travel as far as 60m, although 90% of the wheat pollen remained within 6 m of its source. Other environmental factors like moisture stress may indirectly cause an increase in outcrossing by inducing male sterility.

Moisture stress is another important environmental factor that may contribute to an increased potential for outcrossing in wheat. Water deficit occurs in the plant whenever transpiration exceeds water absorption, because of excessive water loss, reduced absorption, or both. Moisture stress affects almost every aspect of plant growth and development, and influences the anatomy, morphology, physiology and biochemistry of crop plants (Kramer, 1969). Water deficits cause dehydration of protoplasm, associated with a loss of turgor. Both cell division and cell expansion are reduced, resulting in a decrease in plant growth. In addition, cell walls thicken, and there is a greater development of mechanical tissues (May and Milthorpe, 1962).

Numerous studies have been conducted to determine changes in hormone levels during moisture stress in plants. Most of these studies have focussed on abscisic acid (ABA), which has been found to be the hormone most associated with stress in plants (Turner, 1986). Accumulation of ABA has been reported to occur in moisture stressed plants when the leaf water potential falls below a threshold value (Pierce and Raschke, 1980; Quarrie, 1987). Under drought conditions, plants minimize water loss from leaves by closing the stomata, and ABA has been found to play a role in stomatal closing. Mutant plants that lack the ability to make enough ABA are characterized by uncontrolled wilting (Neill and Horgan, 1985; Quarrie, 1982a; Wang et al, 1984). Apart from controlling stomatal conductance, endogenous ABA levels have been reported to affect other processes of plant growth and development, including leaf area, plant height, and root development (Quarrie and Jones, 1977; Biddington and Dearman, 1982). High levels of endogenous ABA have also been found to reduce spikelet number per ear and pollen fertility in wheat (Morgan, 1980). The net effect of high ABA concentration would be a reduced plant size, and reduced floret fertility leading to a reduced demand for water.

The critical stages at which plants are most sensitive to moisture stress have been determined for many crops. Numerous studies indicate that grain crops are particularly sensitive to drought in the two weeks immediately preceding anthesis (Aspinall, et al,1964; Bingham, 1966; Wells and Dubetz, 1970; Fisher, 1973). The reduction in seed set that is observed in grain crops, following periods of moisture stresss at flowering time, has been reported to be mainly a result of a failure in fertilization (Wells and Dubetz, 1970; Innes and Blackwell, 1981; Herrero and Johnson,1981).

In wheat, various studies have indicated that pollen sterility is the main cause of reduced seed set under drought conditions (Morgan, 1980; Bingham, 1966; Saini and Aspinall, 1981). A decrease in grain set in wheat following water deficit occurring during, or a few days prior to pollen meiosis was attributed to male sterility, since female fertility was unaffected (Bingham, 1966).

Other causes of reduced grain yield in moisture stressed wheat have been reported to include limited exsertion of the spike (O'Toole and Namuco, 1983), reduced tillering capacity (Innes et al, 1981), a reduced ratio of spike dry matter to total dry matter at anthesis, and reduced harvest index (Syme, 1969; Fisher et al, 1977; Sharma et al, 1987). Failure of the spike to fully exsert from the flag leaf following stress at flowering was found to cause floret sterility in wheat (O'Toole and Namuco, 1983)

Several researchers have reported differential cultivar response to moisture stress. Saeed and Francis (1984) observed that temperature and rainfall between panicle initiation and anthesis accounted for a significant portion of the cultivar by environment interaction for kernel production and grain yield in sorghum. Wells and Dubetz (1970) showed differential response of barley cultivars to drought at the heading stage. Innes and Blackwell (1981) also observed a differential yield response of two wheat cultivars to pre-anthesis drought resulting primarily from a difference in kernel number.

1.6. HYPOTHESIS

Most of the offtype problems reported in wheat have been in fields of semidwarf cultivars. The offtypes reappeared after roguing suggesting that they were not simply the consequence of seed mixing. Further, some CPS cultivars have been noted to have a high number of sterile florets. Studies have shown that florets open briefly at anthesis, and that male sterile florets open wider and remain open for longer periods than fertile ones (Saran et al, 1971). It has also been suggested that moisture stress affects pollen fertility earlier than it does stigma receptivity, leading to a higher frequency of male sterile florets in wheat.

The hypothesis in this study is that CPS cultivars have a higher frequency of male sterile florets compared to CWRS cultivars, particularly under water stress conditions. Because of this, they have a higher potential for outcrossing than CWRS cultivars. It is proposed that the tall offtypes found in cultivars such as Cutler originate at least in part from outcrossing, consequent to a higher frequency of male sterile florets.

1.7. EXPERIMENTS

To test this hypothesis, several experiments were conducted to study:

(i) the drought tolerance of some CPS and CWRS cultivars

(ii) the frequency of sterile florets in some CPS and CWRS cultivars under well-watered and water stressed conditions

(iii) the pollen viability and stigma receptivity of some CPS and CWRS cultivars under well watered and water stressed conditions

(iv)The potential of a CPS cultivar (Cutler) and a CWRS cultivar (Roblin) to outcross when exposed to external pollen.

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CHAPTER 2

THE EFFECT OF PRE-ANTHESIS MOISTURE STRESS ON FLORET FERTILITY IN SOME SEMIDWARF AND TALL CULTIVARS OF CANADIAN SPRING WHEAT

2.1. INTRODUCTION

Several factors can induce floret sterility in wheat (*Triticum aestivum* L.) They include temperature stress, moisture stress, photoperiod and some forms of nutrient deficiencies (Welsh and Klatt,1971; Entz and Fowler, 1990; Saini and Aspinall, 1981). Many researchers have reported that moisture stress at flowering time results in floret sterility as a consequence of pollen infertility (Bingham, 1966; Saini and Aspinall, 1981). Saini and Aspinall (1981) observed that a large proportion of the anthers on moisture stressed plants were small and shriveled, and did not dehisce normally. Pollen was also devoid of normal cytoplasmic constituents and showed no staining reaction with triphenyl tetrazolium chloride. Cross pollination between moisture stressed and well watered plants revealed that grain set was reduced as a direct consequence of the induction of male sterility by moisture stress, as female fertility was not affected (Bingham, 1966). The induction of male sterility by moisture stress has been attributed to an increase in the concentration of endogenous abscisic acid caused by the drought stress (Morgan, 1980).

Moisture stress can also cause pollen sterility indirectly by causing nutrient deficiency. Low soil moisture was found to enhance copper deficiency in wheat (Grundon, 1991). It has been suggested that copper deficiency interferes with microsporogenesis during meiosis at the early booting stage in wheat, resulting in reduced pollen fertility (Graham, 1976; Owuoche et al, 1994).

Differences in varietal response to environmental stress have been widely reported (Laing and Fisher, 1977; Entz and Fowler, 1990; Naas and Sterling, 1981; Owuoche et al, 1994). In wheat, differential yield response of cultivars to pre-anthesis moisture stress has been reported by many workers (Innes and Blackwell, 1981; Entz and Fowler, 1990). It has also been reported that moisture stress may induce other forms of stress such as copper deficiency (Grundon, 1991), which has been found to induce pollen sterility in wheat (Owuoche, et al, 1994). The effects of copper deficiency on pollen viability, floret fertility and grain yield per plant were found to vary with the cultivar. Naas and Sterling (1981) also reported a varietal difference in root weights of moisture stressed wheat plants. Chapin (1987) suggested that plants with lower developmental rates have a lower demand for nutrients, while Laing and Fisher (1977) suggested that semidwarf wheat cultivars are more susceptible to drought than tall cultivars and may have lower yields in areas with limiting rainfall.

Several methods have been used to evaluate the response of plants to moisture stress. These include germination in osmotic agents like glycol (Powell and Pfeifer, 1956; Johnson and Asay, 1978), observing plant survival following cycles of moisture stress (Todd and Webster, 1965), and water retention of excised leaves (Sandhu and Laude, 1958). The change in membrane permeability following exposure to moisture stress can also be used to estimate drought tolerance in plants (Sullivan, 1971). The leakage of solutes from desiccated tissues has been explained in terms of the changes that take place in the structure of membranes during desiccation (Iljin, 1957; Simon, 1974; Krochko et al, 1978; Bewley, 1979). These alterations are associated with an increase in the activity of hydrolytic enzymes, and to lipid peroxidation (Simon, 1974), which lead to loss in membrane integrity. One of the consequences of the loss of membrane integrity following moisture stress is an increase in cell permeability (Senaratna and McKersie, 1983), and consequently, tests based on the evaluation of membrane resistance could be used in the screening for drought tolerance in crop cultivars (Vasquez-Tello et al, 1990). In vitro desiccation of plant tissues by incubating in a solution of polyethylene glycol (PEG), followed by the measurement of electrolyte leakage into an aqueous medium by electrical conductivity has been found to be useful in estimating drought tolerance in various crops (Blum and Ebercon, 1981; Premachandra and Shimada, 1988; Premachandra et al, 1990).

This study was conducted to investigate the response of some semidwarf cultivars of Canada Prairie Spring wheat (CPS) and some tall cultivars of Canada West Red Spring (CWRS) wheat to periods of moisture stress at flowering time. The focus was on the effect of moisture stress at the booting stage on the frequency of sterile florets in cultivars of the two wheat classes. Drought tolerance of selected cultivars of the two classes was investigated using the electrolyte leakage test. Finally, the cause of floret sterility observed under moisture stress conditions in these cultivars was investigated by studying pollen viability and stigma receptivity.

2.2. MATERIALS AND METHODS

2.2.1. EFFECT OF MOISTURE STRESS ON STERILE FLORET FREQUENCY

Five semidwarf cultivars (Biggar, CDC 1, Cutler, Oslo and Taber), and three cultivars of conventional height (tall) spring wheat (Katepwa, Park and Roblin) were used to study the effects of moisture stress on floret sterility in the two classes of spring wheat. The cultivars were planted in a 3-replicate pot experiment in the greenhouse using 6-inch diameter, 6-inch deep pots. Staggered dates of planting were used so that the cultivars could reach the heading stage at the same date.

The planting medium was a mixture of coarse sand, peat moss and vermiculite in the ratio of 1:2:2 by volume. In about 1000 cm^3 of soil mixture, 270g dolomitic lime, 140g super phosphate (0-20-0), 240g nutricote (14-14-14), 2g Iron chelates and 4g fritted trace elements were added. Six seeds of a cultivar were planted to a depth of 1 cm in each pot and thinning was done one week after emergence to leave four seedlings per pot.

The pots were watered once daily to field capacity. The temperature and photoperiod in the greenhouse were set at 22 ^{o}C /18 ^{o}C and 16 h/8 h day/night, respectively. The lighting was supplemented using high intensity discharge 400W sodium lamps to achieve a light intensity of 450 μE $m^{-2}s^{-1}$ at the pot level.

At the early booting stage, three moisture stress treatments were imposed on the plants: watering to field capacity at (i) 4-day intervals, (ii) 6-day intervals and (iii) 7-day intervals. These intervals were determined on the basis of a preliminary experiment in which unwatered plants did not show symptoms of moisture stress until the fourth day. Daily watering to field capacity was maintained for the control pots. Field capacity in this study refers to the moisture content of the soil medium when it is saturated with water and the excess water is allowed to drain off. The treatments were continued until the flowering process was completed. Thereafter, daily watering to field capacity was resumed for all pots.

After the plants had attained physiological maturity, floret sterility in each cultivar was assessed by counting the number of florets without seed on the main spikes in each pot. This was expressed as a percentage of the total number of florets on these spikes. In order to compare the sterility of the semidwarf and tall classes, the sterility of the cultivars of each class were pooled for each treatment.

During the course of the experiment, pots of each cultivar were weighed at field capacity, and at two days, four days, six days and seven days after watering. The moisture content of the soil in these pots was then calculated based on the weight of air dry soil, to determine rates of water loss from the pots.

2.2.2. ELECTROLYTE LEAKAGE TEST

Drought tolerance of two semidwarf cultivars, Cutler and CDC 1, and two tall cultivars, Katepwa and Roblin, was determined by measuring electrolyte leakage of leaf samples following a period of desiccation using polyethylene glycol-6000 (PEG). This test was conducted using well watered and moisture stressed plants.

Seeds of each cultivar were planted in twelve 6-inch diameter pots in the greenhouse. The planting medium was made by mixing coarse sand, peat moss, soil, and metro mix in equal volumes. All other conditions were similar to those described in section 2.2.1. When the plants reached the early booting stage, two watering treatments were established. Daily watering to field capacity was maintained for six pots of each cultivar while the other six were watered only at 4-day intervals.

Eight days after the initiation of treatments the uppermost fully expanded leaves from each pot were excised, and 5 mm leaf pieces were cut from the middle portion of each leaf. Leaf pieces from each treatment were bulked, and samples of 30 leaf pieces from each bulk were used in a two-replicate electrolyte leakage experiment following the method described by Blum and Ebercon (1981).

The leaf samples were put in small vials and rinsed with three changes of deionized water. The samples were then incubated in 10 ml of either 30% or 50% PEG at 10°C for 24 h. The control samples were incubated in 10 ml of deionized water. After 24 h, all samples were again rinsed with three changes of deionized water, before being incubated in 10 ml of deionized water at 10°C for another 24 h. They were then equilibrated at 25°C before measuring the conductivity of the incubation medium. Following this, the samples were autoclaved for 15 minutes, then equilibrated at 25°C and the conductivity measured again. The electrolyte leakage was determined as the ratio of electrical conductivity of the incubation medium before autoclaving the samples to the conductivity after autoclaving, expressed as percentage.

The injury suffered by each cultivar as a result of desiccation was calculated using the method of Blum and Ebercon (1981) who proposed that:

$$\% \text{ injury}=1-[1-(T1/T2)/1-(C1/C2)] \times 100$$

where T and C refer to the means of treatment and control, respectively and the subscripts 1 and 2 refer to the initial and final conductivities, respectively.

2.2.3. POLLEN FERTILITY EXPERIMENT

To study the effect of moisture stress on pollen fertility, two semidwarf cultivars (Cutler and CDC 1), and two tall cultivars (Roblin and Katepwa) were planted in a 3-replicate experiment in the greenhouse. Both Cutler and CDC 1 have been noted to have a problem of sterile florets. Eight seeds of each cultivar were planted in a 6-inch diameter pot, using a mixture of coarse sand, vermiculite and peat moss in the ratio of 1:2:2 by volume. Staggered planting dates were used to synchronize heading dates. Plants were thinned to four seedlings per pot one week after emergence.

The growing conditions in the greenhouse were similar to those described in section 2.2.1. The pots were watered daily to field capacity until plants reached the early booting stage when two watering treatments were established. In one treatment, daily watering to field capacity was continued while in the second treatment, watering was done only at six-day intervals.

At the onset of flowering, two main spikes were selected from each pot for pollen viability studies. Each selected spike was divided into four equal regions, designated as the basal region, the mid-lower region, the mid-upper region and the distal region. On average, each region contained four spikelets, which were labelled 1, 2, 3, ...etc, starting from the most basal spikelet. The primary, secondary and tertiary florets in each spikelet were designated a, b and c, respectively. In this study, only the primary, secondary and tertiary florets were considered.

During pollen shed, pollen grains from each floret were dusted on a microscope slide and stained with 1% acetocarmine, then examined under the light microscope. In acetocarmine, chromosomes of viable pollen stain pink. Pollen fertility was expressed as the percentage of viable pollen in relation to the number of pollen grains counted.

Pollen fertility of the cultivars under well watered, and moisture stressed conditions were assessed. Most of the florets were found to have either high pollen fertility (>70%) or low pollen fertility (<30%), with few florets having intermediate levels of pollen fertility. This enabled the florets to be classified into those with pollen fertility problem (considered male sterile) and those with good pollen fertility (male fertile). A minimum pollen fertility of 50% was used to separate male fertile from male sterile florets. Most of the florets with low pollen fertility had smaller anthers that often appeared shrivelled.

2.2.4. STIGMA RECEPTIVITY

The effect of moisture stress on the ability of the stigma to receive pollen and enable fertilization to take place were studied in Cutler and Roblin under well watered and moisture stressed conditions. Stigma receptivity was assessed by determining seed set in a pot experiment in the greenhouse. Eight seeds of a cultivar were planted in each pot in a 5-replicate experiment and thinned to four seedlings per pot, one week after emergence. Extra pots of Cutler were planted on staggered planting dates to serve as sources of pollen at flowering. The planting medium was made from coarse sand, vermiculite and peat moss mixed in the ratio of 1:2:2 by volume. The growing conditions in the greenhouse were similar to those described in section 2.2.1. The pots were watered daily to field capacity until the plants reached the early booting stage when two water treatments were established. In one treatment, daily watering to field capacity was maintained while in the other, watering to field capacity was done only at 6-day intervals. The extra pots of Cutler were fully watered to provide a pollen source for crossing.

At flowering, two of the main spikes in each pot were chosen for hand pollination. In these spikes, the pollination process was monitored and any floret that appeared to be lacking pollen was hand pollinated using pollen from the Cutler pollen source. The other two main spikes were left to pollinate naturally.

At maturity, seed set was assessed by determining the percentage of florets with grains in the hand pollinated and non-pollinated spikes in each pot. Seed set in plants under different treatments was compared to determine the effect of moisture stress on stigma receptivity in the two cultivars. Data were analyzed in a factorial randomized complete block design using SAS, and where significant interactions were observed, orthogonal contrasts were done to test differences between means.

2.3. RESULTS AND DISCUSSION

2.3.1. FLORET STERILITY

All the cultivars used in the sterility experiment had some sterile florets even under well watered conditions. Floret sterility for the control treatment ranged from 17% for Biggar to 44% for CDC 1 (Figure 2.1a). The rather high floret sterility noted for most cultivars suggests that there were other factors apart from moisture stress that contributed to floret sterility. One possible cause of high floret sterility in the control plants is nutrient deficiency. Daily watering to field capacity could cause nutrients like copper to leach out, leading to deficiencies. Copper deficiency has been reported to cause pollen sterility (Owuoche et al, 1994). Biggar had the lowest sterility under the control treatment. Owuoche et al (1994) reported that Biggar was the least sensitive to copper deficiency of a number of cultivars studied. The photoperiod used in this experiment (16 h / 8 h day/night) may also have contributed to some floret sterility by reducing pollen viability in some of the cultivars. Welsh and Klatt (1981) observed that pollen viability of some wheat cultivars was reduced as the photoperiod was increased beyond 14 h day^{-1}.

Increase in moisture stress resulted in increased floret sterility in all the cultivars. Such trends have been reported by several researchers (May and Milthorpe, 1962; Fisher, 1973; Morgan, 1980). Analysis of variance using SAS showed a significant cultivar x moisture stress interaction (Table 2.1), indicating that the cultivars differed in their tolerance to moisture stress. When plants were watered at 4-day intervals, the cultivars showed a range in floret sterility from 36% in Biggar to 56 % in Cutler. At this level of stress, the average sterilities for semidwarf and tall cultivars were not significantly different. However, the mean sterility for the semidwarf cultivars at this level of moisture stress was significantly higher ($p=0.01$) compared with the control.

As the watering interval was extended to six, and then to seven days, the floret sterility levels in semidwarf cultivars increased at a higher rate than in the tall cultivars (Figure 2.1b). Under the four moisture regimes, the three tall cultivars exhibited similar levels of sterility, whereas a significant ($p=0.05$) variation was observed within the semidwarf class. When watered at 6-day intervals and the means compared using orthogonal contrasts, for example, two semidwarf cultivars, Biggar and Cutler, showed significantly higher ($p=0.05$) frequencies of sterile florets compared with the tall cultivars while Taber and Oslo had sterility levels comparable to those of the tall cultivars. At the highest level of stress (7-day watering interval), all the semidwarf cultivars had significantly higher ($p=0.01$) levels of floret sterility compared with the tall cultivars. This observation is similar to that made by Laing and Fisher (1977), and McNeal et al (1972) who found that shorter straw wheat yielded lower than the standard height ones when moisture was limiting. Among the cultivars tested, Biggar and Cutler, which were among the cultivars with the lowest sterile floret frequencies under well watered condition, appeared to be the most sensitive to moisture deficit. Their floret sterilities rose sharply with increasing moisture stress, reaching 82 % for Cutler and 74 % for Biggar at the 6-day watering interval.

The average floret sterilities for the semidwarf and the tall cultivars under both the control and the 4-day watering interval treatment were not significantly different. (Figure 2.1b). At 6-day and 7-day watering intervals, the average sterility in semidwarf cultivars was significantly higher (p=0.01) compared to that in tall cultivars. When compared using contrasts, the mean sterilities of the semidwarf and tall cultivars at high moisture stress levels (6-day and 7-day watering intervals) were significantly higher than the respective means at the low stress treatments (control and 4-day watering interval). The difference between the sterility of the semidwarf cultivars at the high stress level and the low stress level was significant at the 1% level while for the tall cultivars, the difference was significant at the 5% level. These results support the findings of Laing and Fisher (1977) who reported that semidwarf cultivars have a higher yield response to rainfall, and consequently, have higher mean yields compared to tall cultivars in high rainfall sites, but are more susceptible to moisture stress.

There was little variation in the rate at which moisture was lost from pots of all cultivars except Biggar (Figure 2.2a and 2.2b). The rate of moisture loss from pots of Biggar was significantly higher (p=0.05) than for the other cultivars. This could possibly be as a result of a greater leaf area as Biggar was noted to be more leafy, although this trait was not measured quantitatively. The moisture content dropped by 3.4% day^{-1} and 2.7% day^{-1} in pots of the semidwarf and tall cultivars, respectively. These rates, however, were not significantly different.

2.3.2. ELECTROLYTE LEAKAGE

The four cultivars showed wide differences in electrolyte leakage under control conditions (Figure 2.3a). Cutler showed the greatest electrolyte leakage, with a conductivity of 56%. The conductivity for Roblin was 43% while CDC 1 and Katepwa had low conductivities of 4.1 and 5.8%, respectively. PEG treatment resulted in increased electrical conductivity in all cultivars, suggesting that there was some damage to the cell membranes that caused them to be more permeable. When incubation was done in 30% PEG, the increases in conductivity for Cutler and Roblin were small and non significant. CDC 1 and Katepwa had about five fold increases in electrolyte leakage, each with conductivity of 27%. However, under this treatment, the electrical conductivities for Cutler and Roblin (66 and 45%, respectively), were still considerably higher than for CDC 1 and Katepwa. Incubation in 50% PEG resulted in large increases in conductivity in all cultivars. Under this treatment, the four cultivars were found to have very similar levels of electrolyte leakage, with conductivities ranging from 77% for Katepwa to 85% for Cutler.

Moisture stressed plants of Cutler, CDC 1 and Katepwa had lower levels of electrolyte leakage compared with well watered plants while Roblin did not show a change in electrolyte leakage in response to moisture stress (Figure 2.3b). Roblin had high electrical conductivities, ranging from 57% for the control to 87% for the samples incubated in 50% PEG. Cutler had the greatest reduction in electrolyte leakage following the moisture stress treatment. Its electrical conductivity ranged from 13.5% for samples incubated in 30% PEG to 32% in samples incubated in 50% PEG.

In order to compare the effect of desiccation on the four cultivars, the level of injury, which is a function of the change in electrolyte leakage following desiccation, was calculated using the method described by Blum and Ebercon (1981). In the well watered plants, incubation in 30% PEG resulted in the same level of injury (25, 24.5 and 23.3%, respectively) in Cutler, CDC 1 and Katepwa (Table 2.1). This treatment had no effect on

Roblin as it showed an injury of only 0.5%. This suggests that out of the four cultivars tested, Roblin is the most resistant to moderate levels of desiccation stress, and possibly to moisture stress. Premachandra and Shimada (1988) reported that the level of injury resulting from desiccation stress was correlated with drought tolerance.

Higher levels of injury (ranging from 64.5% in Cutler to 83.5% in CDC 1) were observed in the samples incubated in 50% PEG, suggesting that this treatment resulted in a greater degree of desiccation in the four cultivars. Leopold et al (1981) found that cowpea (*Vigna sinensis* L.) leaves increased their leakiness in proportion to the extent of desiccation.

In the moisture stressed plants, the injury levels in Cutler, CDC 1, and Katepwa resulting from PEG treatment, were lower than those observed in the well watered plants (Table 2.1). The 30% PEG treatment had no effect on moisture stressed Cutler, and caused injuries of only 11% and 9.5% in CDC 1 and Katepwa, respectively. Moisture stressed Roblin, however, showed an increased level of injury (21%) under this treatment. The 50% PEG treatment resulted in injuries of 18.5% in Cutler, 67% in CDC 1, and 64.5% in Katepwa. These injury levels were considerably lower than those observed in well watered plants, suggesting that moisture stressed plants of Cutler, CDC 1 and Katepwa acclimated to the moisture stress such that they became more resistant to desiccation. A similar observation was made by Blum and Ebercon (1981), who reported that moisture stressed wheat plants showed more desiccation tolerance than well watered plants. Moisture stressed Roblin showed a higher level of desiccation injury compared with well watered Roblin, suggesting that Roblin has less ability to adjust to moisture stress compared with the other cultivars.

Results from this study suggest that of the four cultivars tested, Cutler is the most susceptible to moisture stress under well watered conditions while Roblin is the most tolerant. Moisture stressed Cutler showed the greatest reduction in desiccation injury, suggesting that it had the greatest adjustment to moisture stress. The ability to adjust to stress, however, may not be advantageous in the conditions of this experiment, where the stressing period is short, and occurring at a critical stage. A cultivar such as Roblin that shows inherent tolerance to moderate stress may be able to go through the critical stage before it is affected by the stress and hence will have an advantage over a more sensitive cultivar like Cutler.

2.3.3. POLLEN FERTILITY

Pollen shedding in all the cultivars started in the mid-upper region of the spike and progressed towards the distal and basal ends, with the basal florets being the last to flower. This observation is similar to that made by DeVries (1971). In most spikelets, flowering started with the primary floret and progressed through the secondary and tertiary florets.

Average pollen fertility in the well watered plants ranged from 76 % for Cutler to 95 % for Katepwa (Figure 2.4a). Cutler, CDC 1 and Roblin did not differ significantly for this trait. In most cases pollen fertility in each individual floret was either very high (>70 %) or very low (<30 %). Very few florets showed intermediate levels of sterility. This enabled the classification of the florets into two fertility groups. The florets with pollen fertility greater than 50 % were classified as male fertile while those with lower pollen fertilities were classified as male sterile (Figure 2.4b). Most of the male sterile florets possessed under-developed anthers containing no pollen grains. Possible reductions in pollen and floret fertility in Cutler were masked as a result of high standard errors for the stressed Cutler treatment.

All the cultivars showed differences in pollen fertility in different regions of the spike (Figure 2.5a). Except for CDC 1, fertility was found to be highest in the mid-lower region followed by the mid-upper region then the basal region. The lowest fertilities were observed in the distal region. Briggs (1991) observed seed set in some Canadian wheat cultivars to follow the same trend. He found that after the first three spikelets from the base, seed set decreased from the bottom to the top of the spikes. This suggests that pollen fertility is an important factor in determining seed set in wheat. CDC 1 had a low fertility (60 %) at the basal region but had high fertilities ranging from 88 to 92 % in all the other regions. Katepwa exhibited little variation in pollen fertility between regions, with a range of 89 % (distal) to 100% (mid-lower and mid-upper regions).

Exposing the plants to moisture stress at the heading stage reduced average pollen fertility in all cultivars. (Figures 2.4a and 2.4b). Katepwa showed the greatest drop in fertility, with a drop of about 52 % while Roblin had the least drop in fertility (15 %). Pollen fertilities of Cutler and CDC 1 were reduced by 35 and 44%, respectively. Saini and Aspinall (1981) found that moisture stress in wheat resulted in a large proportion of small shrivelled anthers, which contained non-viable pollen. Several other researchers have reported that moisture stress reduced pollen fertility in wheat, and that there were differences in the varietal responses to moisture stress (Bingham, 1966; Morgan,1980).

Pollen fertility in all regions of the spike was reduced under moisture stress conditions, but standard errors also increased greatly in the moisture stressed treatments (Figure 2.5b). Fertility patterns within the spike did not change in Cutler and Roblin. In Cutler, pollen fertility ranged from 68 % (mid-lower) to 34% (distal), but without significant difference. For Roblin, pollen fertility in the mid-lower region remained quite high (88 %) compared to other regions of the spike, and compared to other cultivars. The basal and distal regions in stressed Roblin had similar pollen fertilities of 54 % and 55 %, respectively.

Moisture stressed Katepwa had the highest pollen fertility (61 %) in the mid-upper region and the lowest fertility (34 %) in the basal region, but these and other positional effects in the spike were not significantly different. Under stress, CDC 1 behaved differently from the other cultivars by showing the highest pollen fertility in the distal region with progressively lower fertilities toward the basal end. It had the lowest fertility (9 %) at the basal end.

During flowering, the pollen that is shed outside the florets tends to fall downwards. This leads to a pollen concentration gradient that increases from regions above the spike to regions below the spike (DeVries, 1972). The existence of such a pollen gradient means that male sterile florets found towards the basal end of the spike are more likely to receive external pollen than those positioned at the distal end of the spike. This suggests that cultivars such as Cutler and Roblin which show the greatest reduction in pollen fertility in the distal region may be more likely to have sterile florets in that region.

2.3.4. STIGMA RECEPTIVITY

In the well watered, non pollinated treatment, both Cutler and Roblin had similar levels of seed set, 77 % and 74 %, respectively (Figure 2.6). A few florets in both cultivars showed signs of lack of fertilization although their stigmas appeared normal and fully grown. When these florets were hand pollinated using pollen from the Cutler pollen source, a small, non significant increase in seed set was observed in each cultivar (to 84 % in Cutler and to 77 % in Roblin).

When the plants were moisture stressed at the heading stage, there was a significant ($p=0.05$) reduction in seed set in both cultivars. Cutler showed a greater sensitivity to moisture stress as its seed set dropped to 21.5 % compared to 59.8 % for Roblin. This represents drops in seed set of 72 % and 19 % for Cutler and Roblin, respectively. The moisture stressed plants, particularly in the case of Cutler, had many florets with apparently normal stigmas but with under developed anthers containing no pollen grains.

Increases in seed set were observed in both cultivars following hand pollination of florets of moisture stressed plants. Analysis of variance performed using the SAS program showed significant interaction between Seed set in Cutler was increased significantly ($p=0.01$) to 62% while in Roblin it was increased to 72%. These represent increases of 195 % in Cutler and 20 % in Roblin. Results from this experiment suggest that the stigmas in the sterile florets were receptive and that most of the reduction in seed set that was observed in moisture stressed plants, particularly in the case of Cutler, was a result of induction of male sterility. Similar results were reported by Bingham (1966) and Saini and Aspinall (1981). These results suggest that under moisture stress, the florets tend to lose pollen viability before losing stigma receptivity. This would lead to a higher frequency of male sterile but female fertile florets, allowing a significant increase in seed set following hand pollination. The fact that a greater increase in seed set was observed in Cutler compared to Roblin means that under moisture stress conditions, Cutler has a higher frequency of male sterile florets than has Roblin. Consequently, moisture stressed Cutler would have a higher potential for outcrossing than Roblin if the two were similarly exposed to external pollen.

Lack of synchrony within the florets in moisture stressed plants could be another cause for reduced seed set. Some of the florets in the moisture- stressed plants appeared to shed pollen before the stigmas were fully expanded. Herrero and Johnson, (1981) reported that poor seed set in moisture stressed maize (*Zea mays* L.) was a result of poor floral synchronization.

CONCLUSIONS

When fully watered, the semidwarf cultivars had a lower frequency of sterile florets, on the average, compared to the tall cultivars. Moisture stress increased the frequency of sterile florets in both semidwarf and tall wheat cultivars. The rate of increase in sterility resulting from increased moisture stress was higher in semidwarf cultivars, resulting in significantly higher ($p=0.01$) sterility in this class at high stress levels.

There were considerable varietal differences in electrolyte leakage following desiccation in 30% polyethylene glycol, with Cutler and Roblin showing much higher levels of electrolyte leakage compared with CDC 1 and Katepwa. When well watered, Cutler was the least tolerant to desiccation injury while Roblin was the most tolerant. Under moisture stress conditions, however, Cutler, CDC 1 and Katepwa showed increased tolerance to desiccation while stressed Roblin had reduced tolerance.

Moisture stressed plants of both Cutler and Roblin had significant ($p=0.05$) reductions in seed set, with a greater reduction in Cutler compared to Roblin. Most of the floret sterility observed in the moisture stressed plants was a result of pollen sterility. Hand pollination of stressed plants resulted in a greater increase in seed set in Cutler than in Roblin indicating that there was a higher frequency of male sterile florets in Cutler. These results suggest that in the presence of an external pollen mass, Cutler has a higher potential for outcrossing compared with Roblin.

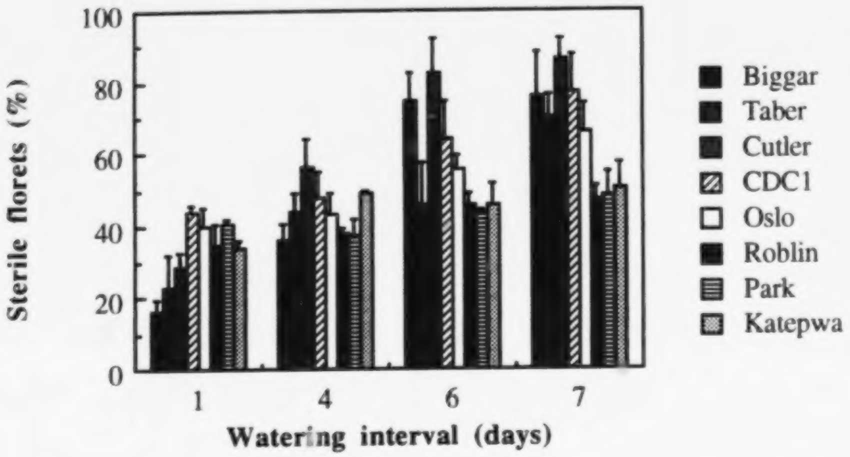

Figure 2.1a. Sterile floret frequencies of five semidwarf wheat cultivars (Biggar, Cutler, CDC1, Oslo and Taber), and three tall cultivars (Katepwa, Park and Roblin) exposed to different levels of pre-anthesis moisture stress (Mean ± SE, n=6).

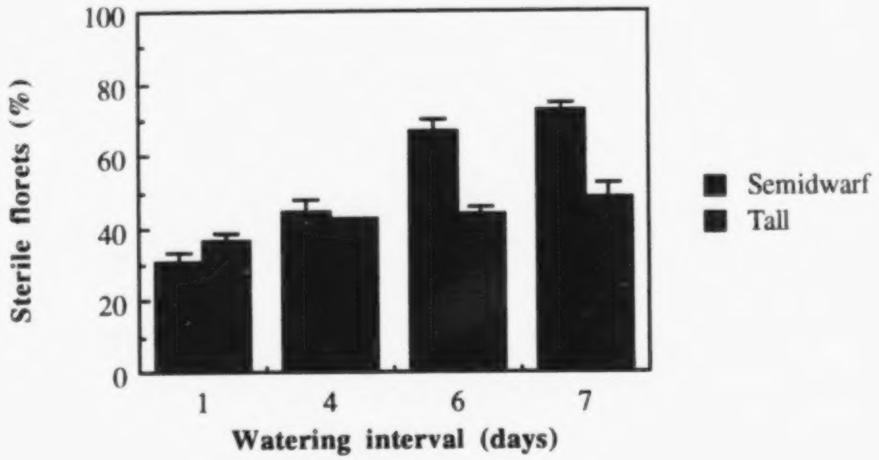

Figure 2.1b. Average sterile floret frequencies for semidwarf and tall wheat cultivars exposed to different levels of pre-anthesis moisture stress. The means are obtained from the sterility frequencies of the cultivars in Figure 2.1a [Mean ± SE, n=30 (semidwarf); n=18 (tall)].

Table 2.1. ANOVA table for the floret sterility experiment involving five semidwarf cultivars (Biggar, Cutler, CDC1, Taber and Oslo) and three tall cultivars (Katepwa, Park and Roblin) under four levels of moisture stress. Data were analyzed in a factorial randomized complete block design using SAS.

Source	df	ms	F
Replication	2	223	1.8 ns
Cultivar	7	736	5.9 **
Moisture level	3	4971	39.8 **
Cultivar x Moisture level	21	332	2.7 **
Error	21	125	

ns non significant.
** significant at p = 0.01.

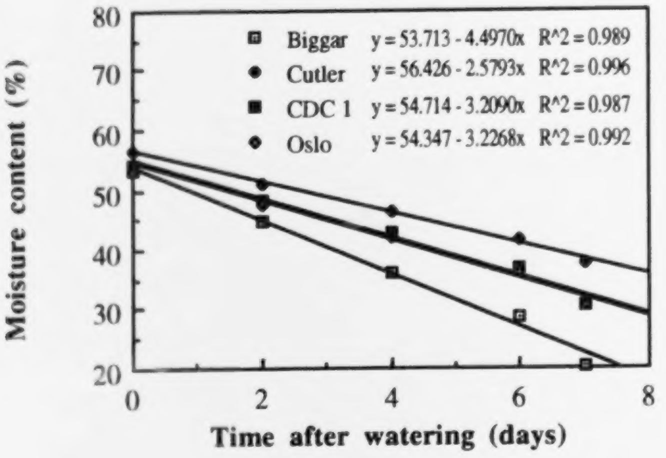

Figure 2.2a. Soil moisture content in pots of four semidwarf cultivars (Biggar, Cutler, CDC1 and Oslo), left un-watered for seven days. Moisture content is based on the weight of air dry soil. The moisture content at time '0' corresponds with the field capacity. The rate of moisture loss from pots of Biggar was significantly higher (p=0.05) than from pots of the other three cultivars.

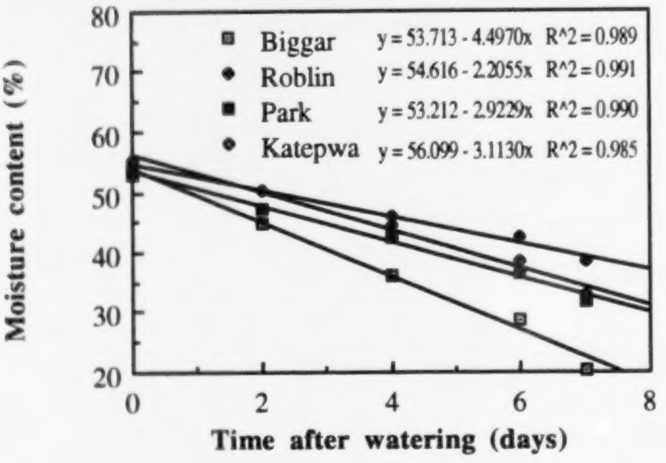

Figure 2.2b. Soil moisture content in pots of three tall cultivars (Katepwa, Park and Roblin) left un-watered for seven days. Moisture content is based on the weight of air dry soil. The moisture content at time '0' corresponds with the field capacity. The graph of the semidwarf cultivar, Biggar is included for comparison.

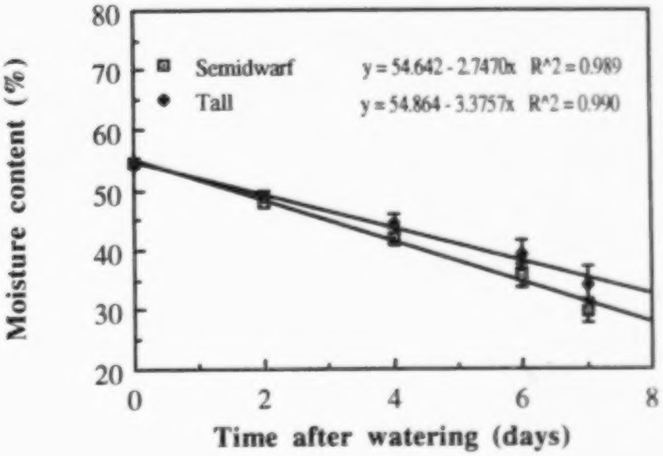

Figure 2.2c Average soil moisture content in pots of the semidwarf cultivars (Biggar, Cutler, CDC1 and Oslo), and the tall cultivars (Katepwa, Park and Roblin), left un-watered for 7 days. The moisture content at time '0' is the field capacity. There was no significant difference in the rate of moisture loss from pots of the two wheat classes.

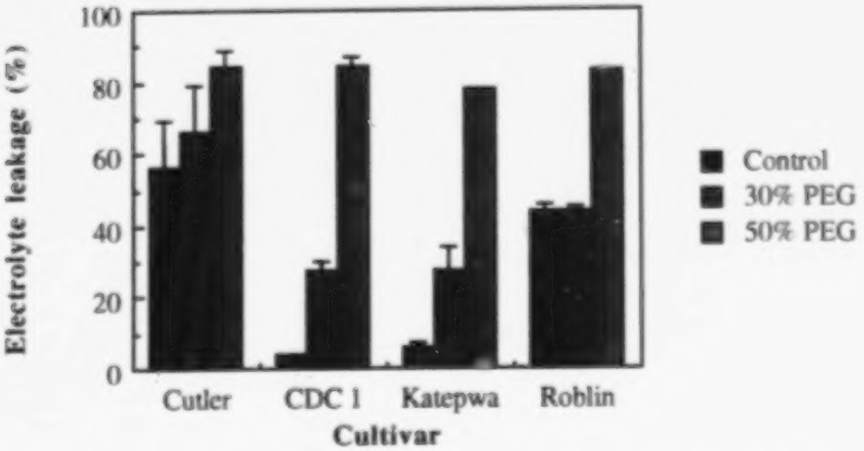

Figure 2.3a. Electrolyte leakage from leaves of well watered plants of Cutler, CDC1, Katepwa and Roblin following incubation in deionized water (Control), 30% PEG and 50% PEG. The leakage was determined by measuring the electrical conductivity of the aqueous medium in which the samples were incubated after desiccation (Mean ± SE, n=2).

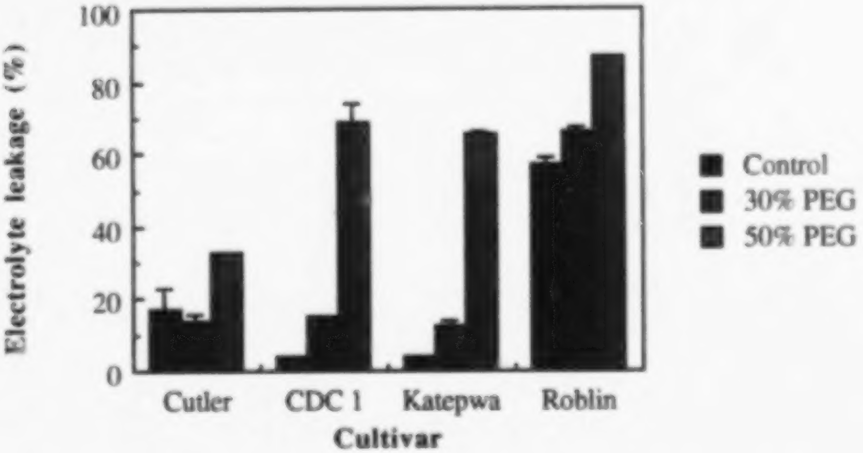

Figure 2.3b. Electrolyte leakage from leaves of moisture stressed plants of Cutler, CDC1, Katepwa and Roblin following incubation in deionized water (Control) 30% PEG, and 50% PEG. Leakage was determined by measuring the electrical conductivity of the aqueous medium in which the samples were incubated after desiccation (Mean ± SE, n=2).

Table 2.2. Percent injury in leaf samples of Cutler, CDC 1, Katepwa and Roblin incubated for 24 h in 30% and 50% PEG. Means are from two replicates. Standard errors are shown in parentheses.

	WATERED		STRESSED	
CULTIVAR	30% PEG	50% PEG	30% PEG	50% PEG
Cutler	25.0 (8.0)	64.5 (0.5)	0.0 (4.5)	18.5 (6.5)
CDC 1	24.5 (2.5)	83.5 (2.5)	11.0 (0.0)	67.0 (6.0)
Katepwa	23.3 (8.0)	76.0 (0.0)	9.5 (1.5)	64.5 (0.5)
Roblin	0.5 (2.5)	70.0 (1.0)	21.0 (6.0)	69.5 (1.5)

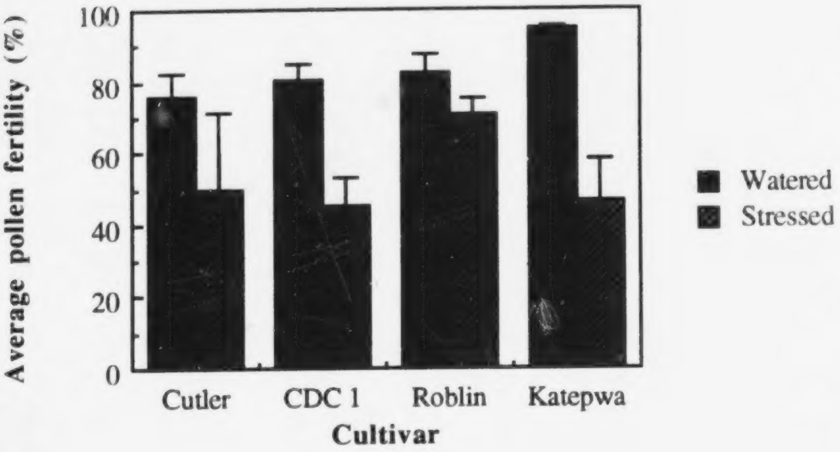

Figure 2.4a. Average pollen fertility per spike in moisture stressed and well watered plants of Cutler, CDC1, Katepwa and Roblin (Mean ± SE, n=6).

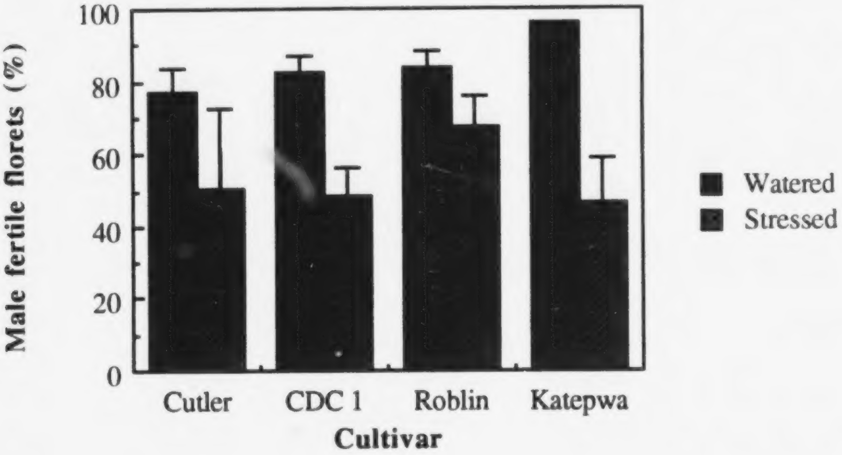

Figure 2.4b. Proportion of florets with more than 50% pollen fertility in moisture stressed and well watered plants of Cutler, CDC1, Katepwa and Roblin (Mean ± SE, n=6).

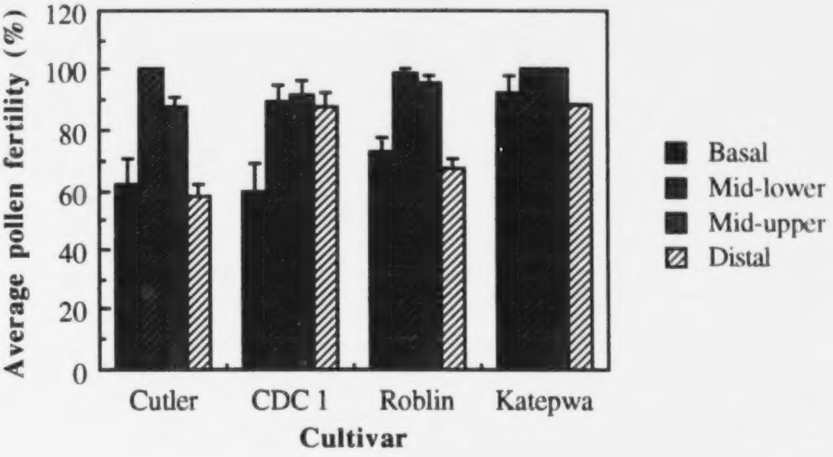

Figure 2.5a. Average pollen fertility per region of spike in well watered plants of Cutler, CDC1, Katepwa and Roblin. Fertility was determined by staining pollen in 1% acetocarmine and counting the number of viable pollen (Mean ± SE, n=6).

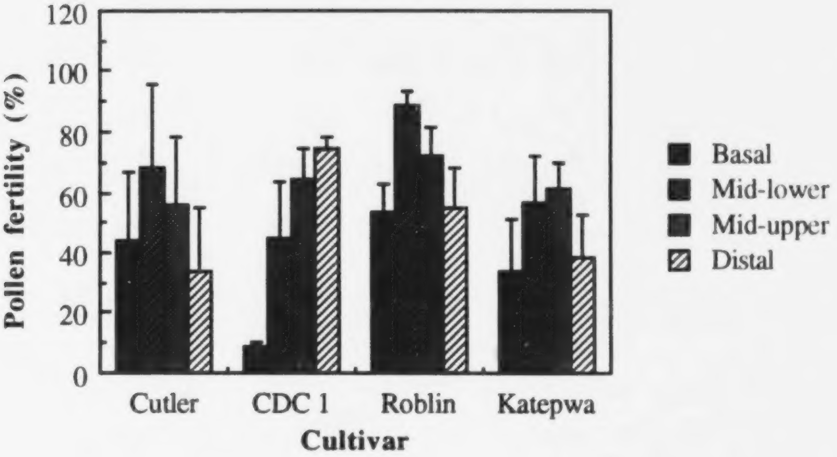

Figure 2.5b. Average pollen fertility per region of spike in moisture stressed plants of Cutler, CDC1, Katepwa and Roblin. Fertility was determined by staining pollen in 1% acetocarmine (Mean ± SE, n=6).

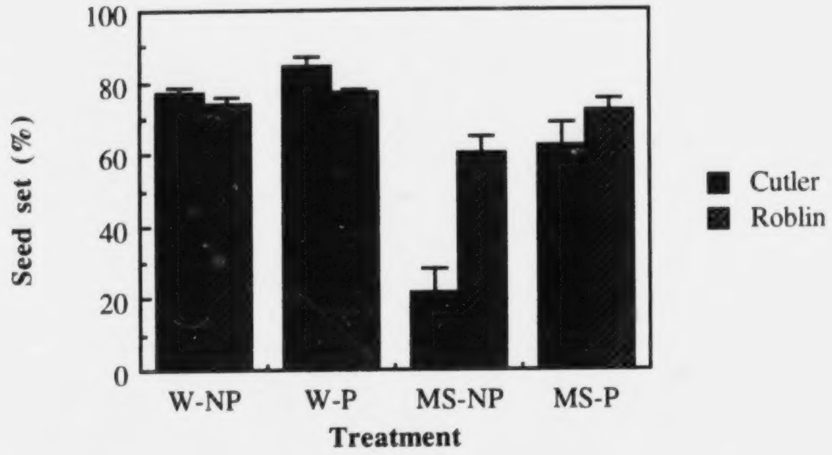

Figure 2.6. Stigma receptivity measured as % seed set in moisture stressed and well watered Cutler and Roblin, with and without hand pollination. W-NP=watered, non pollinated; W-P=watered, pollinated; MS-NP=moisture stressed, non pollinated; SP=moisture stressed, pollinated. (Mean ± SE, n=10).

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CHAPTER 3

3. FLORET STERILITY AND OUTCROSSING IN A SEMIDWARF AND A CONVENTIONAL HEIGHT SPRING WHEAT CULTIVAR

3.1. INTRODUCTION

Wheat (*Triticum aestivum* L.) is considered a self pollinating crop because of its floral structure and flowering behaviour (Leighty and Sando, 1924). Wheat florets are enclosed by two glumes, the palea and the lemma, and open only briefly at anthesis. External pollen is, therefore, normally excluded from the florets, leading to very low levels of cross pollination. The extent of natural outcrossing in cultivated varieties of wheat has been reported to range from 0-4% (Heyne and Smith, 1967). Rigorous preventative measures are not usually considered in wheat during seed production or seed multiplication because of the perceived low risk of outcrossing. Many seed production guidelines recommend isolation distances between a seed field and other wheat fields to be wide enough to sufficiently prevent mechanical mixing. The Canadian seed production regulations, for example, require an isolation distance of only 3 m for wheat (Anon. 1994). Similarly, the FAO wheat seed production guidelines require the seed field to be isolated from all other fields of wheat, or crop species with similar seed size, by a distance adequate to prevent mechanical mixing, or to be separated by a physical barrier like a fence (Anon. 1993).

Outcrossing, however, may be a significant problem in seed production. There have been concerns regarding the appearance of tall offtype plants in some semidwarf wheat cultivars (Worland and Law, 1985; Storlie and Talbert, 1993). Tall offtypes have also been observed in most of the Canada Prairie Spring (CPS) wheat cultivars so far released (Briggs, Pers. comm.). Offtypes may occur in wheat cultivars for several different reasons. These include accidental mixing of seed, occurrence of volunteer plants in the field, some genetic abnormalities, and outcrossing. Offtypes that reoccur after rogueing suggest that segregation for the offtype plants was still occurring. Progeny of offtypes found in the CPS cultivar Cutler, for example, segregated for both height and awnedness (Briggs, Pers. com.). It is unlikely that these offtypes resulted from seed mixing or from volunteer plants, and, therefore probably arose from outcrossing.

Storlie and Talbert (1993) found that seed protein electrophoresis of offtypes resulting from aneuploidy gave electrophoregrams similar to those of the pure parents. When polyacrylamide gel electrophoresis of gliadins was performed on offtypes found in Cutler, the banding patterns were found to be different from those of Cutler (data not shown), suggesting that the offtypes were not caused by aneuploidy. These observations leave outcrossing as the most likely cause of the offtypes in Cutler.

Studies, particularly in the field of hybrid seed production, have shown that it is possible to have high levels of cross pollination in wheat. Seed set as high as 75% on male sterile parents is common in hybrid production fields (Virmani and Edwards, 1984). The extent of cross pollination is influenced by both plant and environmental factors. The important plant factors include the extent of floret opening, the size of the stigma, the duration of stigma receptivity, exsertion of the stigma, anther size and filament length (De Vries, 1971; Virmani and Edwards, 1984). Significant varietal differences have been observed in most of the floral structure attributes which are important in cross pollination (Cahn, 1925; Kherde et al, 1967; De Vries, 1974a, 1974b; Virmani and Edwards, 1984).

Male sterile plants were found to have a longer period of floret opening compared to fertile plants (Saran, et al, 1971). The opening of florets at anthesis is controlled by lodicules located at the base of the ovary (Percival, 1921; Kadam, 1933). When the lodicules increase in turgidity, they push apart the lemma and palea, causing the florets to open. McNeal and Ziegler (1975) reported that lodicules from male sterile or emasculated florets were significantly heavier, wider, and thicker than those from male fertile florets.

Moisture availability is one of the environmental factors that could influence cross pollination in wheat. Moisture stress at the heading stage has been found to increase the frequency of male sterile florets in wheat (Bingham, 1966; Morgan, 1980; Saini and Aspinall, 1981). Adequate moisture is also required for the proper functioning of the lodicules (McNeal and Ziegler, 1975).

In a greenhouse experiment to study the effects of moisture stress on the frequency of sterile florets in some semidwarf (CPS) and tall Canada Western Red Spring (CWRS) wheat cultivars, it was found that under moisture stress conditions, CPS cultivars have significantly higher frequency of sterile florets than CWRS cultivars (Chapter 2). The results from a stigma receptivity study suggested that moisture stressed Cutler has a significantly higher frequency of male sterile florets than moisture stressed Roblin (Chapter 2). This would suggest that moisture stressed Cutler has a higher potential for outcrossing than moisture stressed Roblin.

In the present study, outcrossing potentials for moisture stressed Cutler and Roblin were studied to determine whether the higher frequency of male sterile florets observed in Cutler could lead to increased outcrossing. This was done by exposing moisture stressed plants of both cultivars to pollen from a marker line, P8901. This is a tall, awnless line with a black chaff colour. The black chaff colour is a dominant marker that would allow the assessment of outcrossing in the F_1 plants.

3.2. MATERIALS AND METHODS

The potentials for outcrossing in a semidwarf cultivar, Cutler, and a conventional height (tall) spring wheat cultivar, Roblin, were compared in a greenhouse experiment by exposing moisture stressed plants of the two cultivars to external pollen from a marker stock during flowering, and then determining the extent of outcrossing in each cultivar.

Cutler is an awned, very early maturing, semidwarf spring wheat with special adaptation to the Parkland region of the Western Prairies (Briggs et al., 1991). It belongs to the Canada Prairie Spring (CPS) wheat class. Roblin is an awnless, tall spring wheat cultivar belonging to the Canada West Red Spring (CWRS) wheat class. It is a high-protein, Marquis-type quality, early maturing and rust-resistant cultivar developed for the eastern prairies of Canada (Campbell and Czarnecki, 1987).

The marker stock used was a line designated P8901, obtained from Dr. R. M. De Pauw (Agriculture and Agri-Food Canada, Swift Current, Saskatchewan). This is a conventional height Hard Red Spring wheat with a black chaff colour. The black chaff colour is a dominant marker (De Pauw, pers. comm. to Briggs) and enables determination of outcrossing in the F_1 plants.

Staggered planting dates were used for Cutler and Roblin to synchronize heading dates. Pots of P8901 marker stock, which is later maturing than both Cutler and Roblin, were planted at several dates, starting ten days before the planting of Roblin.

Planting was done in a 4-replicate experiment in the greenhouse using 6-inch diameter, 6-inch deep pots. Eight seeds of a cultivar were planted in each pot and seedlings were thinned to four per pot, one week after emergence. The planting medium was a mixture of coarse sand, vermiculite and peat moss in a volume ratio of 1:2:2. The temperature and photoperiod in the greenhouse were set at $22^oC/18^oC$ and 16h /8h day/night, respectively. The lighting was natural daylight supplemented with artificial light from high intensity discharge 400W sodium lamps to give a light intensity of approximately 450 $\mu E\ M^{-2}S^{-1}$ at the pot level.

All the pots were watered daily to field capacity until the plants reached the early booting stage. Field capacity refers to the moisture condition of the soil when it is saturated with water and the excess water is allowed to drain. At the early booting stage, three treatments were imposed on Cutler and Roblin. The first treatment (control) was a continuation of the daily watering to field capacity. The second was a moisture stress treatment in which watering to field capacity was done only at 6-day intervals. The third treatment was watering to field capacity only at 6-day intervals, and exposing the moisture stressed plants to marker pollen from P8901, throughout the flowering period.

Only the main spikes were considered in this experiment. These spikes were bagged before the start of flowering. Then, throughout the flowering period, spikes from treatment 3 were uncovered, once daily, and exposed to pollen from P8901. This was done by picking mature anthers that were ready to dehisce from P8901 using a pair of tweezers, and dehiscing them about 15 cm above the spikes of Cutler and Roblin such that the pollen fell freely onto the spikes. Florets on the spikes of Cutler and Roblin receiving this treatment were neither emasculated nor artificially opened. To ensure a steady supply of pollen throughout the flowering period, some pots of P8901 were transferred to a cooler chamber (15°C) to slow down the flowering process and extend anthesis. When flowering was completed, the spikes were uncovered and daily watering to field capacity was resumed in all pots.

When the plants had reached physiological maturity, seed set was determined on the main spikes of plants in each of the three treatments. This was expressed as the percentage of florets that had grains, in relation to the total number of florets in these spikes. The changes in seed set following exposure of stressed plants to external pollen was taken as an indicator of outcrossing potential.

When the plants were ready for harvesting, the seed from the spikes of Cutler and Roblin, which were moisture stressed and exposed to marker pollen during flowering were harvested singly, noting their positions on the spike. These spikes were divided into four approximately equal regions, designated as the basal region, the mid-lower region, the mid-upper region and the distal region. The spikelets in each region were numbered 1, 2, 3 etc., starting from the most basal spikelet, and the florets in each spikelet were designated a, b, and c for the primary, secondary and tertiary florets, respectively.

Progeny tests were then used to determine the level of outcrossing that occurred following exposure to marker pollen. This was done by planting individual seeds in 5-inch diameter pots in the greenhouse and observing the expression of the black chaff colour and other phenotypic characters of P8901 such as tallness, late maturity and awnlessness in the F_1 plants. The medium used for planting, and the growth conditions in the greenhouse were similar to those described above for the parents.

3.3. RESULTS AND DISCUSSION

3.3.1. FLORET STERILITY AND OUTCROSSING BASED ON SEED SET

Under well watered conditions (control), both Cutler and Roblin showed low levels of floret sterility, exhibiting seed sets of 76% and 83%, respectively (Figure 3.1). When the cultivars were moisture stressed by increasing the watering interval to six days, significant reductions in seed set occurred in both cultivars. However, the reduction was more pronounced in Cutler with a drop in seed set of 89% compared to 49% in Roblin.

When moisture stressed plants of Cutler and Roblin were exposed to pollen from the marker cultivar, seed set increased from 9% to 48% in Cutler and from 42% to 49% in Roblin. The increase in seed set was significant ($p=0.05$) in Cutler but it was not significant in Roblin.

The effect of moisture stress on pollen fertility has been studied by many researchers (Bingham, 1966; Morgan, 1980; Saini and Aspinall, 1981). It has been reported that when wheat plants are exposed to moisture stress at the heading stage, pollen fertility is affected before stigma receptivity, and that the floret sterility that is induced by moisture stress is caused mainly by pollen sterility.

The results from this experiment show that with respect to floret sterility, Cutler is more sensitive to moisture stress than Roblin. An increase in seed set following exposure of moisture stressed plants to external pollen indicates that there are some florets with functional stigmas but which fail to set seed because of a lack of viable pollen. The large increase in seed set (more than four fold) that was observed in moisture stressed Cutler following exposure to external pollen suggests that male sterility was the main cause of floret sterility and reduced seed set in this cultivar. In Roblin, there was a small, non significant increase in seed set following exposure to external pollen suggesting that pollen sterility was not the main cause of reduced seed set, or that the entry of the external pollen into the florets was limited.

Observation of moisture stressed plants of Cutler and Roblin also showed that the florets tend to open wider and for longer periods compared to those of well watered plants (Figure 3.2a). This could be an indication that moisture stressed plants had a higher frequency of male sterile florets. Saran et al. (1971) reported that male sterile, or emasculated florets opened wider and for a longer period than fertile florets. Florets of moisture stressed Cutler appeared to open wider than those of moisture stressed Roblin, suggesting that florets of moisture stressed Cutler have better chances of receiving external pollen and hence a higher potential for outcrossing. This could be one of the reasons why there was a significant increase in seed set in moisture stressed Cutler following exposure to marker pollen. In an earlier experiment (Chapter 2), it was found that hand pollination of moisture stressed plants of Cutler and Roblin resulted in a significant increase in seed set in Cutler and a non significant increase in Roblin. Assuming that the additional seed set that is realized when moisture stressed Cutler and Roblin are exposed to pollen is a result of outcrossing, the results from this experiment suggest that under moisture stressed conditions, Cutler has a higher potential for outcrossing than Roblin.

3.3.2. DETERMINATION OF OUTCROSSING BASED ON THE PHENOTYPE OF F_1 PLANTS

The black-chaff marker line used in this experiment differed from Cutler in many phenotypic characters including height, awnedness and maturity, all of which would make it easier to identify outcrosses in the F_1 plants. Apart from the black chaff colour, the only other character in which the marker line differed with Roblin was maturity. To be able to compare outcrossing levels in Cutler and Roblin effectively, it was, therefore, important that the black chaff colour be expressed in all the outcrossed F_1 plants.

Several F_1 plants (progeny of Cutler and Roblin plants that were moisture stressed and exposed to pollen from P8901) expressed the black chaff colour after they had attained physiological maturity. Among the plants that showed the black chaff colour in the F_1 plants derived from Cutler, most were semidwarf, awned and slightly later maturing than Cutler (Figure 3.2b), and a few were tall, awnless and later maturing. There were a few of these F_1 plants, however, that were tall, awnless and later maturing than Cutler, but did not express the black chaff colour (Figure 3.2c). This suggests that some F_1 outcrosses may not be identified using the black chaff marker alone. This could lead to under estimation of outcrossing in a cultivar such as Roblin, that has no other markers.

In the F_1 plants derived from Roblin, only those exhibiting the black chaff colour could clearly be recognized as outcrosses. All the plants were awnless and similar in maturity. Most of the plants showing the black chaff colour were the same height with Roblin, while a few of the plants were slightly taller. (Figure 3.2d).

The intensity of the black chaff colour in the F_1 plants obtained from crossing P8901 with either Cutler or Roblin was not as strong as in P8901 but was intermediate between the two parents (Figures 3.3a and 3.3b). This suggests an incomplete dominance of the black chaff colour. Piech and Evans (1979) attributed a reduction in intensity of a purple grain colour in F_2 seed to incomplete dominance of the character.

Cutler showed a higher frequency of offtype plants (considered to be outcrosses), compared with Roblin. When outcrossing was determined using expression of the black chaff colour, height, awnedness and maturity, the proportion of outcrossed F_1 plants in Cutler was found to be 78% compared to 52% for Roblin.

The potential for outcrossing per region of spike in moisture stressed Cutler and Roblin was examined by comparing seed set distribution across the spikes with the distribution of outcrossed seed. The spikes of the two cultivars from which seed for this experiment were obtained had similar seed distribution patterns. In both cultivars, most of the seed was found in the mid-upper region, followed by the mid-lower region, the distal region and the basal region (Figure 3.4a). The regions that had higher seed sets also had more outcrosses. The pattern of distribution of outcrosses in the spikes of Cutler and Roblin differed. Cutler had a very low seed set in the basal region and subsequently no outcrosses were found in this region. In the remaining regions, Cutler showed a more uniform distribution of outcrosses compared with Roblin (Figure 3.4b). In Roblin, most of the outcrossed seed (56.9%) occurred in the mid-upper region, followed by the distal region (25.3% of the outcrosses). There was little outcrossing in the mid-lower and the basal regions with only 10.7 and 7.0% of the outcrosses, respectively.

Most of the outcrossing in Roblin was confined to the distal and the mid-upper regions. The mid-lower region had a very low frequency of outcrosses. In the pollen fertility experiment (Chapter 2), it was also found that pollen fertility in the mid-lower region of moisture stressed Roblin was significantly higher than in the other regions. This further supports the hypothesis that a reduction in pollen fertility leads to an increase in the potential for outcrossing.

In order to compare the frequency of outcrossing in the different regions of the spikes of Cutler and Roblin, an outcrossing index, which is a ratio of the number of outcrossed seeds found in each region to the number of seeds found in that region was calculated (Table 3.1). In Cutler, the basal region, which contained a small proportion of the seed (7%), had no outcrossed seed. The other three regions had high frequencies of outcrossed seed with the outcrossing index ranging from 0.74 in the mid-lower region to 0.91 in the mid-upper region. In Roblin, the mid-lower region had a low frequency of outcrossed seed compared with the mid-upper and the distal regions. The outcrossing index ranged from 0.15 in the mid-lower region to 0.76 in the distal region.

3.4. CONCLUSIONS

Moisture stress significantly reduced seed set in both Cutler and Roblin. On exposure of moisture stressed plants of Cutler and Roblin to external pollen, an increase in seed set was observed in both cultivars, suggesting that a shortage of viable pollen is one of the factors that causes reduced seed set in the moisture stressed plants. The increase in seed set from applying supplemental pollen, and the expression of the black chaff colour in many F_1 plants indicates that outcrossing occurred in both cultivars.

The increase in seed set was large and significant in Cutler compared to a marginal increase in Roblin. Cutler also showed a higher proportion of outcrosses in the F_1 plants. The results from this study suggest that moisture stressed Cutler has a higher potential for outcrossing compared with moisture stressed Roblin. The smaller extent of outcrossing observed in Roblin compared with Cutler could have resulted from a lower frequency of male sterile florets in Roblin or a limited access of external pollen into its florets.

One problem with the black chaff marker used in this study is that it was not always clearly expressed in the F_1 outcrosses. It is, therefore, possible that outcrossing especially in Roblin, was under estimated since there were no other reliable phenotypic markers for identifying outcrosses.

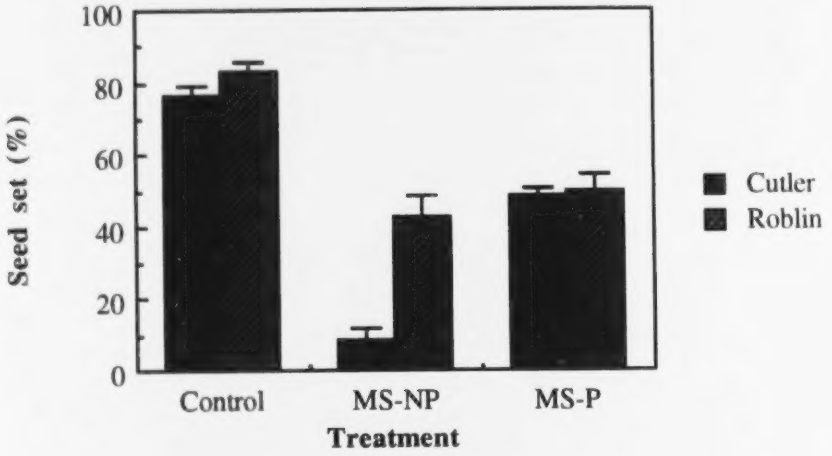
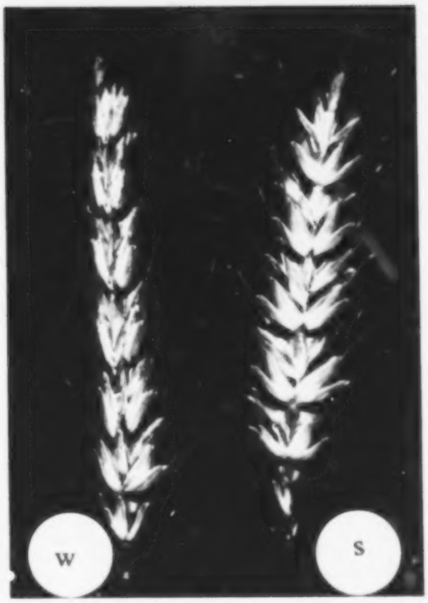


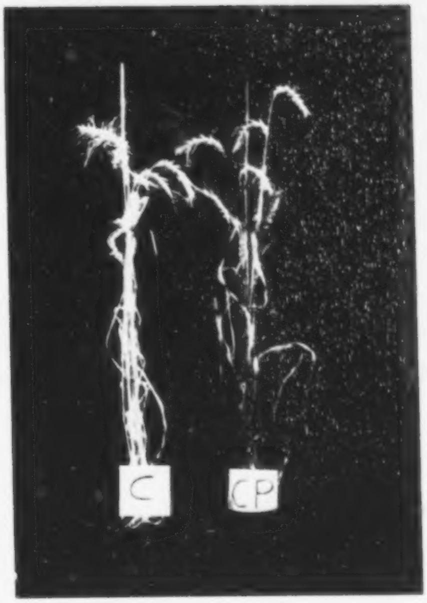
Figure 3.1. Effect of moisture stress on seed set in Cutler and Roblin, and the response of the two cultivars to the presence of an external pollen mass. Control: watered daily to field capacity; MS-NP: moisture stressed (watered only at 6-day intervals) without exposure to external pollen; MS-P: moisture stressed, with a daily exposure to external pollen at flowering. (Mean ± SE, n=16).

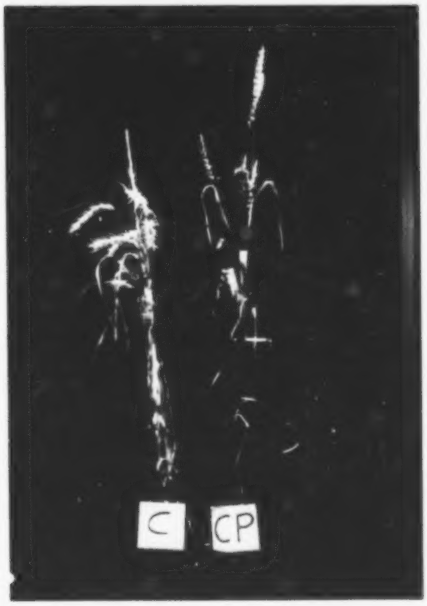
Figure 3.2. (i) Extent of floret opening in well watered (w) and moisture stressed (s) plants of Cutler. (ii) Cutler (C) and an awned F_1 plant from a Cutler x P8901 cross (CP), showing differences in maturity. (iii) Cutler (C) and a tall, awnless, white-chaff F_1 plant (CP) from a Cutler x P8901 cross. (iv) Roblin (R) and a F_1 plant (RP) from a Roblin x P8901 cross showing differences in chaff colour and height.

I

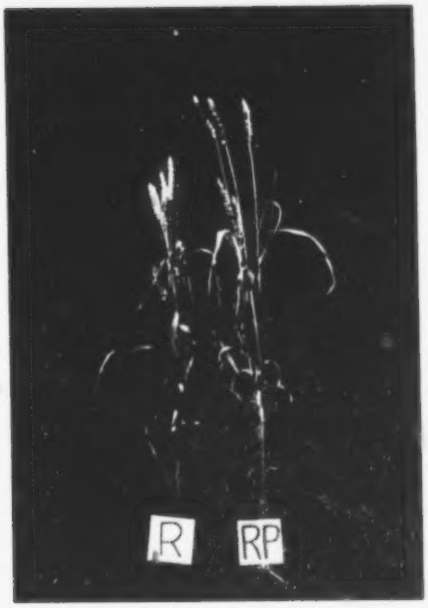


II





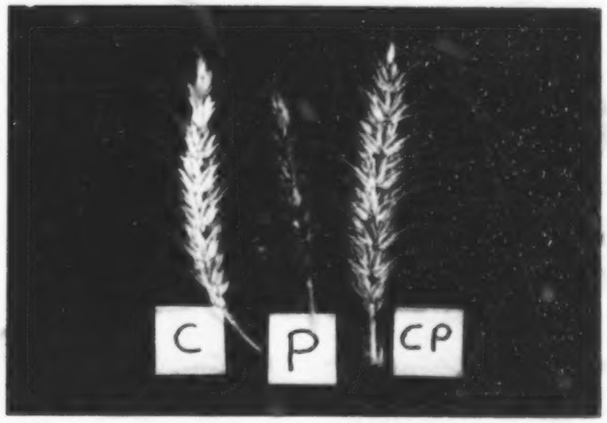
III

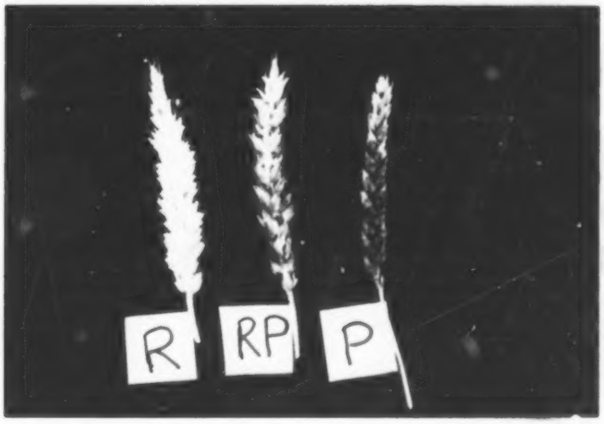


IV

Figure 3.3a. Cutler (C), P8901 (P) and their F_1 (CP), showing a reduced intensity of the black-chaff colour in the F_1.

Figure 3.3b. Roblin (R), P8901 (P), and their F_1 (RP), showing an intermediate intensity of the black-chaff colour in the F_1.


C
P
CP


R
RP
P

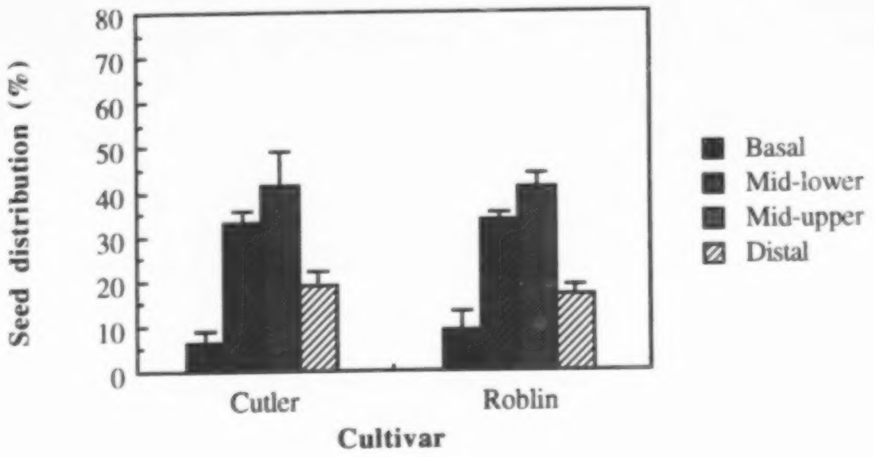


Figure 3.4a. Seed distribution across the four regions of spike in Cutler and Roblin plants that were moisture stressed at the heading stage and exposed to pollen from P8901. (Mean ± SE, n=4).

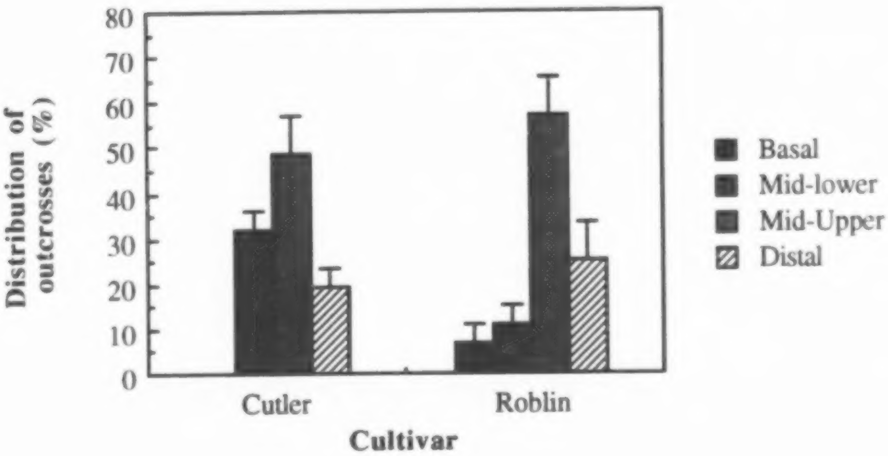


Figure 3.4b. Distribution of outcrossed seeds across the four regions of spike in moisture stressed plants of Cutler and Roblin following exposure to P8901 pollen. Outcrossing was determined by observing the phenotype of the F_1 plants. (Mean ± SE, n=4).

Table 3.1. Frequency of outcrosses per region of spike in moisture-stressed plants of Cutler and Roblin exposed to pollen from P8901. Outcrosses were determined based on expression of the phenotypic characters of P8901 in the F_1 plants. The outcrossing index is the ratio of the number of outcrossed seeds found in a region to the number of seeds found in that region. Standard errors are shown in parentheses.

CULTIVAR	Region	Number of seeds per region [A]	Proportion of seed per region (%)	Number of outcrosses per region [B]	Proportion of outcrosses per region (%)	Outcrossing index [B]/[A]
Cutler	Distal	4.0 (1.2)	20.5	3.0 (0.8)	20.5	0.75
	Mid-upper	7.5 (1.0)	38.5	6.8 (0.8)	46.6	0.91
	Mid-lower	6.5 (1.3)	33.3	4.8 (1.0)	32.9	0.74
	Basal	1.5 (0.6)	7.7	0.0 (0.0)	0.0	0.00
		19.5 (4.2)		14.6 (2.6)		
Roblin	Distal	3.3 (0.5)	16.8	2.5 (0.9)	26.0	0.76
	Mid-upper	7.8 (0.8)	39.8	5.3 (0.5)	55.2	0.68
	Mid-lower	6.5 (1.0)	33.2	1.0 (0.4)	10.4	0.15
	Basal	2.0 (0.9)	10.2	0.8 (0.5)	8.3	0.40
		19.6 (3.1)		9.6 (0.8)		

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CHAPTER 4

4. DETERMINATION OF OUTCROSSING IN CUTLER AND ROBLIN WHEAT CULTIVARS BY ELECTROPHORESIS OF SEED STORAGE PROTEINS

4.1. INTRODUCTION

The extent of outcrossing in moisture stressed plants of Cutler and Roblin wheat (*Triticum aestivum* L) cultivars that were exposed to pollen from a marker line, P8901 was determined using electrophoresis of seed proteins. Cutler is a semidwarf cultivar of the Canada Prairie Spring (CPS) wheat class, which has been observed to have a problem with offtype plants. Roblin is a conventional height (tall) cultivar of the Canada West Red Spring (CWRS) wheat class. P8901 is a tall, awnless line with a black chaff colour, a single gene dominant trait that was obtained from Dr. R. M. De Pauw (Agriculture and Agri-Food Canada, Swift-Current, Saskatchewan).

In an earlier experiment, outcrossing in Cutler and Roblin was determined by examining the phenotype of the F_1 plants, (Chapter 3), and Cutler was found to have a higher potential for outcrossing compared with Roblin. However, the results were complicated by the observation that some of the outcrosses apparently did not express the black chaff colour.

Gel electrophoresis of seed proteins has become one of the most important techniques for laboratory cultivar characterization in wheat (Cooke, 1984). The three main protein fractions of wheat grain (albumin, gliadin and glutenin) have been assessed in terms of their suitability for varietal identification (Wrigley et al, 1982; Garcia-Olmedo et al, 1982). The gliadin proteins (alcohol soluble fraction) have been found to be the most suitable since they show more polymorphisms and are readily extracted and fractionated (Wrigley et al, 1982). Seed storage proteins have been found to be polymorphic with respect to either size, charge, or both. These differences can be visualized on electrophoregrams, and are characteristic to the wheat genotype (Coulson and Sim, 1964; Lee and Ronalds, 1967; Wrigley, 1970; Wrigley and Shepherd, 1974). The use of protein electrophoresis to characterize wheat cultivars is favoured over the use of morphological characters since the electrophoregrams are not affected by environmental conditions (Zillman and Bushuk, 1979; Lookhart and Finney, 1984; Cooke, 1984). Zillman and Bushuk (1979) found that gliadin electrophoregrams were not affected by several environmental factors including methods of grain sample preparation, storage time for ground grain or gliadin extracts, and extraction time. Lookhart and Finney (1984) reported that neither severe frost when the grain was immature nor pre-germination of mature wheat seed for up to 44 h caused significant changes in gliadin electrophoregrams. The electrophoregram is a stable genotypic character, and can be used to identify the cultivar if appropriate protein extracts are used.

Electrophoresis of reduced glutenin sub-units has also been used to characterize wheat cultivars (Ng et al, 1988). Because the synthesis of glutenin is under a different genetic control than that of gliadin (Lawrence and Shepherd, 1980, Dachkevitch et al, 1993), glutenin analysis should provide different information about the genotype.

Seed proteins have been separated using three main types of gels. These are acidic starch gels, acidic polyacrylamide gels, (acid-PAGE), and sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) (Cooke, 1984). Starch gels were the first to be used to separate gliadins. However, polyacrylamide gels are now preferred since the resolution in starch gels is influenced by the stirring and the heating conditions of the starch slurry, leading to lowered reproducibility (Wrigley et al, 1982).

When cultivars with polymorphic seed proteins are crossed, the inheritance of genes can be studied by observing the expression of the polymorphic bands in the subsequent generations (Konarev et al, 1979). This offers the possibility of using protein electrophoresis to study outcrossing between cultivars with polymorphic proteins. In the present study, outcrossing in Cutler and Roblin was determined by examining the F_1 seed for the presence of protein bands present in the pollen donor (P8901), and absent in Cutler or Roblin. Electrophoregrams obtained from acid-PAGE of gliadins and SDS-PAGE of reduced glutenin sub-units were examined.

4.2. MATERIALS AND METHODS

4.2.1. ACIDIC POLYACRYLAMIDE GEL ELECTROPHORESIS (acid-PAGE) OF GLIADINS

4.2.1.1. PLANT MATERIAL

Gliadin proteins were extracted from seed of Cutler, Roblin, P8901, and F_1 seed from Cutler x P8901 and Roblin x P8901 crosses, and separated using acid-PAGE to study the polymorphisms of the three parents as well as to determine whether the polymorphic bands of the pollen donor (P8901) could be detected in the F_1 seed. Gliadin proteins were also extracted from F_1 seed obtained from plants of Cutler and Roblin that were moisture stressed at the heading stage and exposed to pollen from P8901. Protein extraction and electrophoresis procedure closely followed that described by Ng et al (1988).

4.2.1.2. GLIADIN PROTEIN EXTRACTION

Four seeds from each of Cutler, Roblin, P8901, and the crosses Cutler x P8901 and Roblin x P8901 were crushed into fine flour, and a 40 mg sample was transferred to a 1.5 ml centrifuge tube. These bulked samples were used as the reference samples during electrophoresis of proteins from single seeds. Single seeds from Cutler and Roblin plants that were moisture stressed and exposed to pollen from P8901 were crushed and the flour was also transferred to 1.5 ml centrifuge tubes. Gliadin proteins were extracted by adding 80 µl of 70% ethanol to each tube, mixing, and letting to stand for 30 min. The tubes were centrifuged for 2 min. at 13000 rpm using a microcentrifuge, and 25 µl of the supernatant was diluted with an equal volume of electrode buffer (0.25% w/v aqueous aluminium lactate adjusted to pH 3.1 with lactic acid), containing 40% w/v sucrose and 0.5% w/v methyl green dye. The pellet was saved to be used for glutenin extraction.

4.2.1.3. GEL POLYMERIZATION

The glass plates of the Hoefer Scientific SE 600 vertical slab gel electrophoresis unit were assembled according to the manufacturer's instructions. 1.5 mm thick, 6% gels were polymerized by adding 0.5 ml of 1% hydrogen peroxide to 40 ml of gel solution (6% w/v acrylamide, 0.3% w/v bis[1], 0.1% w/v ascorbic acid and 0.0015% w/v ferrous sulphate in electrode buffer) and quickly pouring in between the glass plates. Combs were inserted in the gel solution, and polymerization was allowed to continue for 30 min. The wells were then filled with electrode buffer before loading the samples.

4.2.1.4. ELECTROPHORESIS

Electrophoresis was run using protein samples from Cutler, P8901, Roblin and the F_1 crosses (Cutler x P8901 and Roblin x P8901). Samples from Cutler and Roblin plants that were exposed to pollen from P8901 were used in subsequent runs to determine the outcrosses. In each gel, samples of parents (Cutler or Roblin, and P8901) were included for reference.

Eight µl alliquots were loaded in the wells and the gels were transferred into the lower buffer chamber, which contained 4.5 l of electrode buffer. 500 ml of electrode buffer was poured into the upper buffer chamber, and the power was connected such that the upper electrode was positive and the lower one was negative.

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[1] N, N'-methylene bisacrylamide

Electrophoresis was carried out for 3 h at a constant current of 30 mAmp per gel. During electrophoresis, the gels were cooled by circulating water at 20°C in the lower buffer chamber. After electrophoresis the gels were stained overnight in Coomassie stain (5 ml of 0.5% Coomassie Brilliant Blue R-250 solution, 12.5 ml of 100% trichloroacetic acid, and 112.5 ml of water), then rinsed several times with distilled water, and photographed on a light box using type 55 black and white, instant polaroid film. The extent of outcrossing was determined by counting the number of F_1 seeds that expressed the polymorphic bands specific to P8901.

4.2.2. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

4.2.2.1. GLUTENIN EXTRACTION

The glutenin proteins were extracted from the pellets that remained after gliadin extraction, by adding 1 ml of extraction solution (24 ml H_2O, 10.2 ml extraction buffer stock solution[2], 1.8 ml 2-mercaptoethanol) into each tube, mixing, and letting to stand for 2 h. The tubes were heated in a boiling water bath for 2.5 min, cooled to room temperature, centrifuged for 1 min at 1300 rpm, and alliquots from the supernatant used for electrophoresis.

4.2.2.2. GEL POLYMERIZATION

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[2] 24 ml water, 12.5 ml 0.5M Tris-HCl (pH=6.8),30 ml glycerol

Glass sandwiches for the Hoefer Scientific SE 600 electrophoresis unit were assembled according to manufacturer's instructions. 1.5 mm thick, 17% separating gels were polymerized by mixing 16.9 ml water, 56.6 ml 30% acrylamide/bis solution (aqueous solution containing 29.2 % w/v acrylamide and 0.8% w/v bis), 25 ml separating gel buffer (1.5 M Tris[3]- HCl, pH 8.8), 1 ml 10% SDS, 500 µl 10% ammonium persulphate, and 50 µl TEMED[4]. The mixture was poured between the glass plates and left for 1 h to polymerize. The stacking gel (12.2 ml water, 5 ml 0.5 M Tris-HCl pH=6.8, 200 µl 10% w/v SDS, 2.6 ml 30% acrylamide/bis solution 100 µl 10% w/v ammonium persulphate, and 20 µl TEMED) was polymerized on top of the separating gel.

4.2.2.3. ELECTROPHORESIS

The wells in the stacking gel were filled with electrode buffer (72.1 g glycine, 15.2 g Tris, and 5 g SDS in 5 litres of water, pH=8.3), and 10 µl alliquots of protein extracts were loaded in each well. A high molecular weight marker (MW-SDS-200, from Sigma) was loaded in one lane of each gel to enable estimation of the molecular size of the proteins. Also, samples of Cutler or Roblin, and P8901 were included in each gel to act as reference standards. The gels were transferred into the lower buffer chamber containing 4.5 litres of electrode buffer, then 500 ml of buffer was poured in the upper buffer chamber, and the power was connected such that the positive electrode was to the bottom of the gel and the negative electrode was to the top. Electrophoresis was performed at a constant current of 5 mAmp per gel until the samples reached the separating gel, then the current was was raised to 10 mAmp per gel and electrophoresis was continued for 18 h. Throughout electrophoresis, the gels were cooled by continuous circulation of water at 20°C in the lower buffer chamber.

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[3] Tris (hydroxymethyl) aminomethane

[4] N, N, N', N'-tetramethyl ethylene diamine

The gels were stained overnight using Coomassie Brilliant Blue G-250 staining solution, then rinsed, and left overnight in distilled water on a shaker The stained gels were placed on a light box and photographed using type 55 black and white polaroid film. Outcrossing was determined by counting the F_1 seeds that showed polymorphic bands specific to P8901.

4.3. RESULTS AND DISCUSSION

4.3.1 GLIADIN PROTEIN ELECTROPHORESIS

Most of the bands observed in the gliadin electrophoregrams of Cutler, Roblin and P8901 were common to the three cultivars (Figure 4.1). This similarity could be a result of selection for certain characteristics. Metakovsky et al (1993) suggested that gliadin bands that are common to cultivars of a region may serve as markers of important characteristics of wheat in that region. Most of the polymorphic bands were found among the slow moving gliadins.

Since acid-PAGE separates gliadins on the basis of charge and size, the molecular weights of the bands could not be determined. Individual bands in this experiment were described by their relative mobilities in the gel. For discussion purposes, a band of intermediate size, and common to the three cultivars was arbitrarily assigned a relative mobility of 50 (Figure 4.1a). This band was then used as a reference to calculate the relative mobilities of the other bands in the gel.

Six bands were polymorphic between Cutler and P8901. These were bands with relative mobilities of 17.3, 19.3, 20.8, 22.8, 26.7 and 29.7 The bands with relative mobilities of 17.3 and 20.8 were present in Cutler and absent in P8901 while the other 4 bands were present in P8901 and absent in Cutler (Figure 4.1a, Table 4.1). The F_1 seed from Cutler x P8901 cross expressed most of the bands from the two parents, although the intensities for some bands were not as strong as in the parents (Figure 4.1a). The fact that the F_1 seed expresses the bands of both parents indicates that gliadin electrophoregrams can be used to determine outcrossing levels in Cutler exposed to pollen from P8901.

Roblin and P8901 showed seven polymorphic bands. These were bands with relative mobilities of 17.3, 20.8, 25.7 and 33.2, which were present in Roblin but absent in P8901, and bands with relative mobilities of 19.3, 22.8, and 26.7, which were present in P8901 but absent in Roblin (Figure 4.1a, Table 4.1). F_1 seed of Roblin x P8901 cross also expressed the bands of the two parents, suggesting that it was possible from electrophoresis of gliadins to identify outcrosses in Roblin that resulted from outcrossing with P8901 (Figure 4.1a).

4.3.2 SDS-PAGE OF GLUTENIN SUB UNITS

In a preliminary SDS-PAGE of glutenin proteins from Cutler, Roblin, P8901, and the F_1 crosses (Cutler x P8901 and Roblin x P8901), where glutenin was extracted from fresh samples (samples from which proteins had not previously been extracted), some polymorphic bands were observed in the three cultivars and the F_1 crosses. P8901 expressed one band (approx. 180 kD) that was absent in both Cutler and Roblin, and another (approx. 155 kD) that was absent in Roblin (Figure 4.1b; Table 4.2). The two bands were present in the F1 seed, but their intensities were much lighter. The intensity of the 180 kD band was too light to enable it to be used as a marker for outcrossing.

When glutenin was extracted from the pellets remaining after gliadin extraction, however, the polymorphic bands from P8901 (180 kD and 155 kD) could not be detected in any of the F_1 seed. This possibly resulted from a lower concentration of glutenin in the samples. Outcrossing in Cutler and Roblin was, therefore, determined based on the results of the gliadin electrophoresis alone.

4.3.3 OUTCROSSING BASED ON GLIADIN ELECTROPHOREGRAMS

The outcrosses in Cutler exhibited many bands that are present in P8901 but absent in Cutler. The most prominent of these bands were those with relative mobilities of 22.8, 26.7 and 29.7 (Figures 4.2a and 4.2b). Expression of two polymorphic bands (relative mobilities of 22.8 and 26.7), enabled the detection of outcrosses in Roblin (Figures 4.3a and 4.3b). These bands were present in P8901, and absent in Roblin. There was a wide variation in the extent of outcrossing in spikes of the two cultivars as indicated by the number of seeds that expressed the polymorphic bands of P8901. Roblin in particular showed outcrossing levels ranging from 0% in some spikes to as high as 90 % in others. On average, Cutler showed a higher frequency of outcrosses (84%) compared with Roblin (37%). These results agree with those obtained using phenotypic markers (Chapter 3), which found 78% outcrossing in Cutler compared to 52% in Roblin. This suggests that moisture stressed Cutler has a higher frequency of male sterile florets compared with moisture stressed Roblin, or that the external pollen had a limited access into the florets of Roblin.

The distribution of outcrosses in Cutler had the same pattern as the seed distribution across the spike (Figures 4.4a and 4.4b). Most of the seed (44 %) occurred in the mid-upper region followed by the mid-lower region (33%), the distal region (16%), and the basal region (7%). Similarly, most of the outcrosses (44 %) were found in the mid-upper region with the mid-lower, distal and basal regions having 27, 21 and 8% of the outcrosses, respectively.

In Roblin, as in Cutler, most of the seed was found in the mid-lower and the mid-upper regions, which had 38 and 37 % of the total seed, respectively. The distribution of outcrosses in Roblin was skewed towards the distal end of the spike. The distal region had the highest proportion of outcrosses (48 %), followed by the mid-upper region (34 %), the mid-lower region (14 %) and the basal region (3 %). The outcrossing patterns across the spikes of Cutler and Roblin in this experiment were similar to those observed using phenotypic markers, indicating that gliadin electrophoregrams can be used to study outcrossing in the two cultivars.

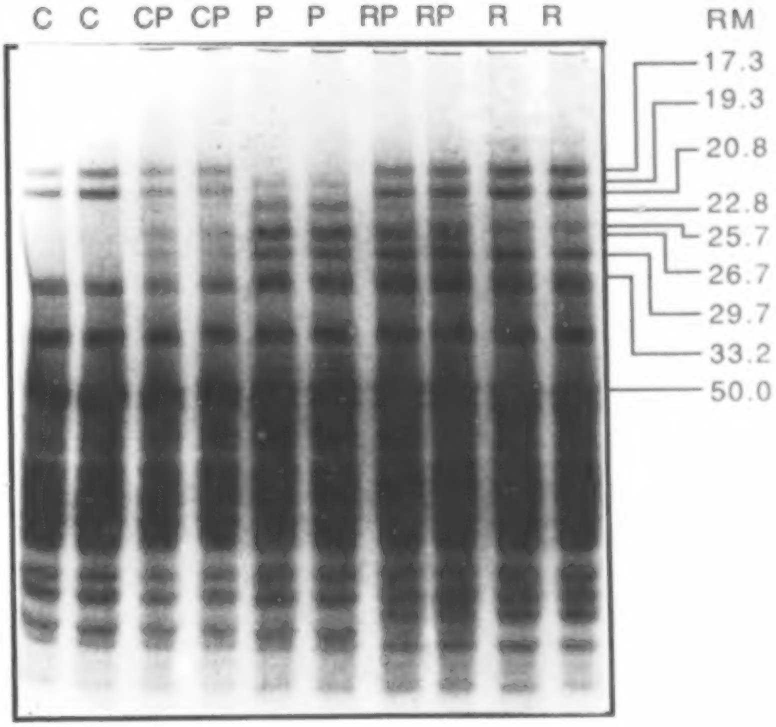
An outcrossing index, calculated as the ratio of the number of outcrossed seeds in a region to the number of seeds in that region was developed to enable a comparison of the frequency of outcrosses in the different regions of the spike (Table 4.3). From the outcrossing index, it was observed that Cutler has a high frequency of outcrosses in all the four regions with the outcrossing index ranging from 0.73 in the mid-lower to 1.00 in the distal and basal regions. Roblin showed a wider variation in the frequency of outcrosses in the different regions of spike compared with Cutler. The basal and the mid-lower regions in Roblin both showed very low frequencies of outcrosses with outcrossing indices of 0.15 and 0.22, respectively. These results suggest that water stress greatly affected floret fertility in all the regions of spike in Cutler while the effect in Roblin was confined mainly to the distal and the mid-upper regions.

4.4 CONCLUSIONS

Acidic polyacrylamide gel electrophoresis of gliadin proteins showed polymorphisms among Cutler, Roblin, and P8901. The F_1 crosses, Cutler x P8901 and Roblin x P8901 exhibited some of the polymorphic bands of P8901, thereby allowing the determination of outcrossing from the electrophoregrams. There was a wide variation in the extent of outcrossing in each spike. However, on the average, moisture stressed Cutler showed a higher potential for outcrossing compared with moisture stressed Roblin. The highest frequency of outcrosses in both cultivars occurred in the distal region. The frequency of outcrosses in Cutler, however, was more uniform across the different regions of the spike, whereas in Roblin, there was a wide variation in frequency, with the distal region having a very high frequency, and the basal and mid-lower regions having very low frequencies.

Figure 4.1a. Electrophoregram from acid-PAGE of gliadins from Cutler (C), Cutler x P8901 (CP), P8901 (P), Roblin x P8901 (RP), and Roblin (R). Gliadins were separated in a 6 % polyacrylamide gel and stained in Coomassie. Relative mobilities (RM) of some of the polymorphic bands are shown.

Figure 4.1b Electrophoregram from SDS-PAGE of glutenin sub units from Cutler (C), Cutler x P8901 (CP), P8901 (P), Roblin x P8901 (RP) and Roblin (R), separated in 17 % polyacrylamide gel and stained in Coomassie.


C
C
CP
CP
P
P
RP
RP
R
R
RM
17.3
19.3
20.8
22.8
25.7
26.7
29.7
33.2
50.0

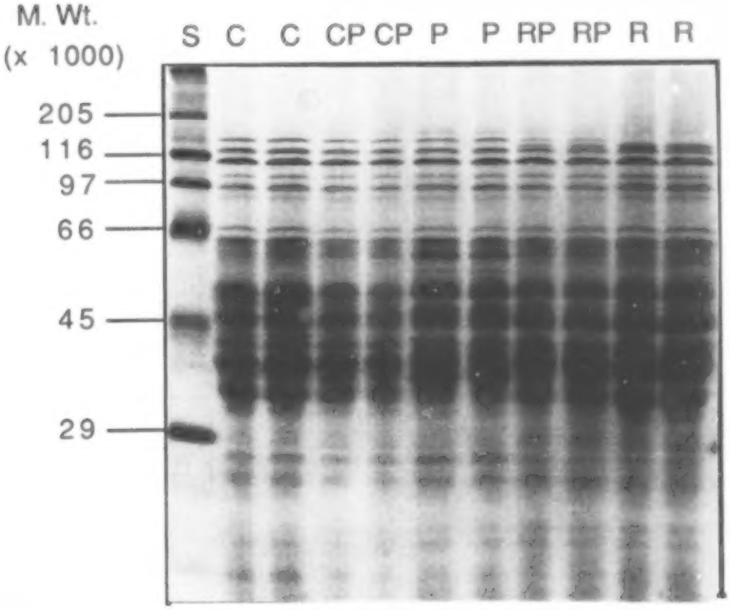

M. Wt.
(x 1000)
S
C
C
CP
CP
P
P
RP
RP
R
R
205
116
97
66
45
29

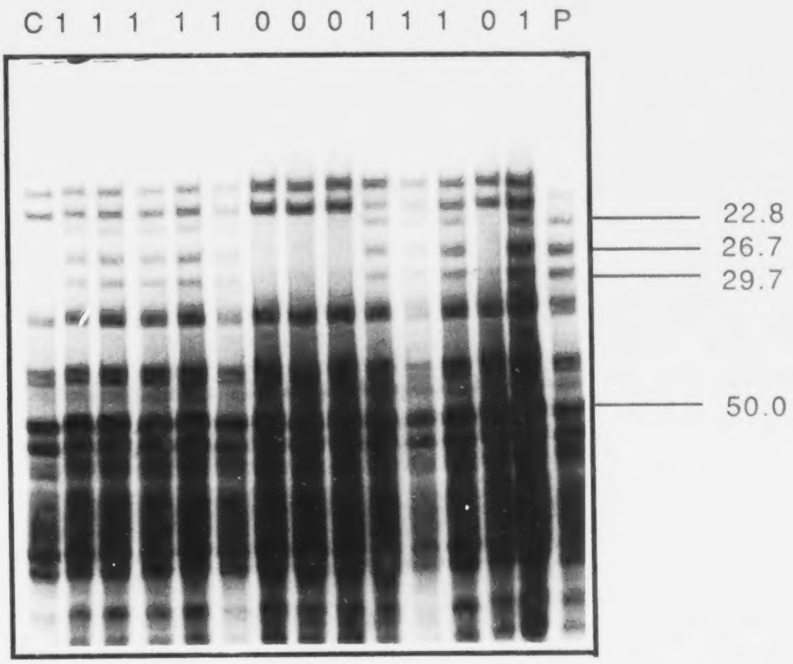
Table 4.1. Relative mobilities of the polymorphic gliadin bands of Cutler, P 8901, Roblin, and the F_1 crosses (Cutler x P8901 and Roblin x P8901), separated by means of electrophoresis in 6% polyacrylamide gel. (+) indicates the presence of the band while (-) indicates its absence.

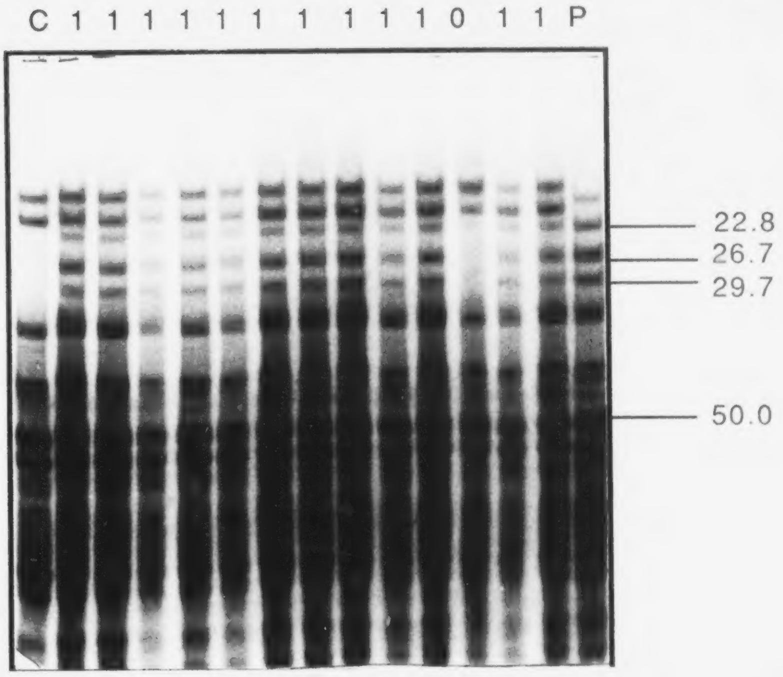
CULTIVAR/	Band (R M)							
CROSS	17.3	19.3	20.8	22.8	25.7	26.7	29.7	33.2
Cutler	+	-	+	-	-	-	-	-
Cutler x P8901	+	-	+	+	-	+	+	-
P8901	-	+	-	+	-	+	+	-
Roblin x P8901	+	-	+	+	+	+	+	+
Roblin	+	-	+	-	+	-	+	+

Table 4.2 Polymorphic glutenin protein bands of Cutler, P 8901, Roblin, and the F_1 seeds of the crosses (Cutler x P 8901 and Roblin x P 8901) obtained from SDS-PAGE. The glutenins were reduced using 2-mercaptoethanol, and their sub units separated in 17 % polyacrylamide gel.

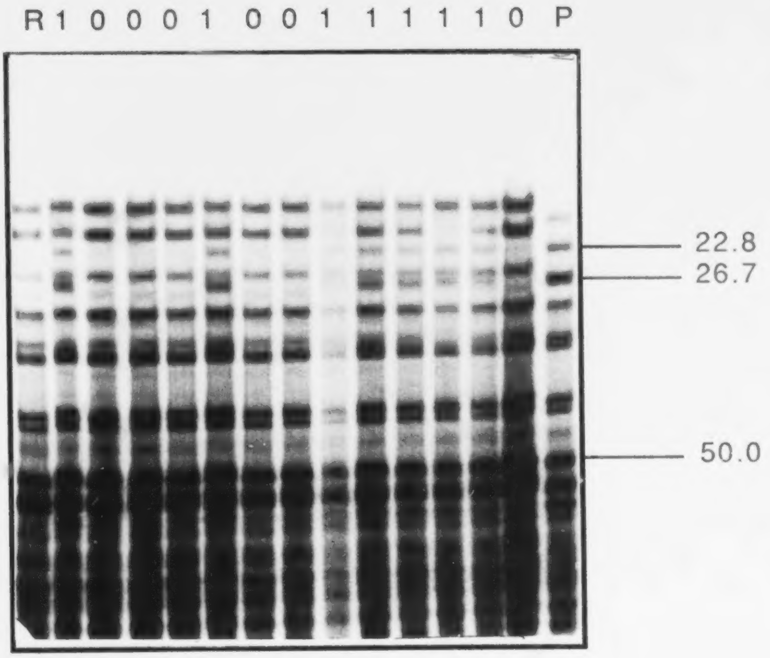
CULTIVAR/ CROSS	Molecular weight (kD)			
	125	140	155	180
Cutler	+	-	+	-
Cutler x P 8901	+	-	+	+
P 8901	+	-	+	+
Roblin x P8901	+	+	+	+
Roblin	+	+	-	-

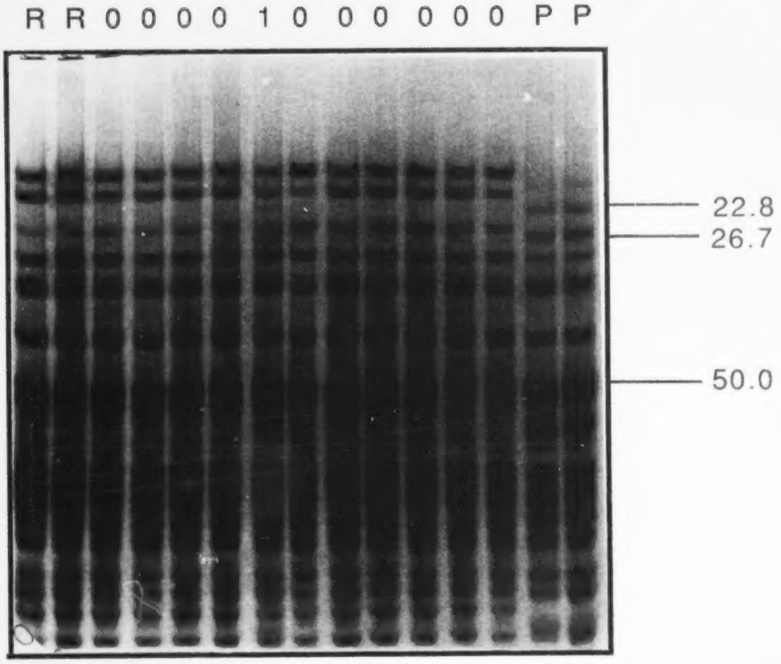
Figures 4.2.a and 4.2.b. Acid-PAGE electrophoregrams of gliadin proteins from Cutler (C), P8901 (P), and F_1 seeds from Cutler plants that were moisture stressed, and exposed to pollen from P8901. Relative mobilities of the polymorphic bands of P8901 are shown. Lanes of F_1 seeds exhibiting bands of P8901 are labelled (1) while lanes of F_1 seeds with banding patterns similar to Cutler's are labelled (0).


C1 1 1 1 1 0 0 0 1 1 1 0 1 P
22.8
26.7
29.7
50.0


C 1 1 1 1 1 1 1 1 1 1 0 1 1 P
22.8
26.7
29.7
50.0

Figures 4.3a and 4.3b. Acid-PAGE electrophoregrams of gliadin proteins from Roblin (R), P8901 (P), and F_1 seeds of Roblin plants that were moisture stressed and exposed to pollen from P8901. Relative mobilities of some polymorphic bands of P8901 are shown. The lanes of F_1 seeds that showed the polymorphic bands of P8901 are labelled (1) while those of F1 seeds with banding patterns similar to Roblin's are labelled (0).


R 1 0 0 0 1 0 0 1 1 1 1 1 0 P
22.8
26.7
50.0


R R 0 0 0 0 1 0 0 0 0 0 0 P P
22.8
26.7
50.0

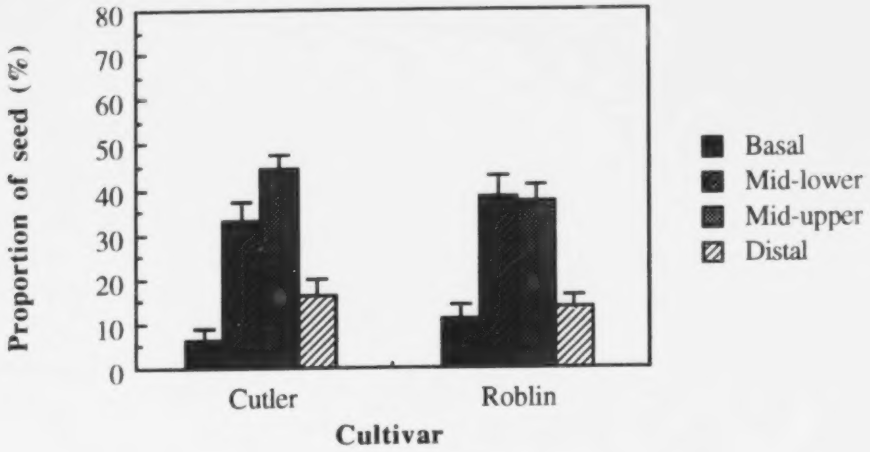


Figure 4.4a. Seed distribution across the different regions of the spike in plants of Cutler and Roblin that were moisture stressed and exposed to pollen from P8901 at flowering. The values represent the number of seeds per region expressed as a percentage of the number of seeds per spike. (Mean ± SE, n=8).

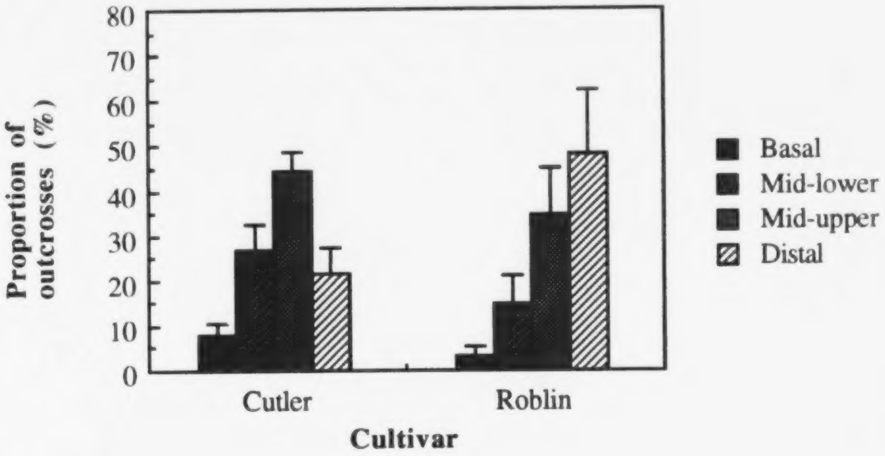


Figure 4.4b. Distribution of outcrosses in the different regions of spike in plants of Cutler and Roblin that were moisture stressed and exposed to pollen from P8901 at flowering. The values represent the number of outcrossed seeds per region expressed as a percentage of the number of outcrosses per spike. (Mean ± SE, n=8).

Table 4.3. Frequency of outcrosses per region of spike in moisture stressed plants of Cutler and Roblin exposed to pollen from P8901. Outcrosses were determined based on expression of the polymorphic gliadin bands of P8901 in the F_1 seed. The outcrossing index is the ratio of the number of outcrossed seeds found in a region to the number of seeds found in that region. Standard errors are shown in parentheses.

CULTIVAR	Region	Number of seeds per region [A]	Proportion of seed per region (%)	Number of outcrosses per region [B]	Proportion of outcrosses per region (%)	Outcrossing index [B]/[A]
Cutler	Distal	2.3 (0.5)	15.8	2.3 (0.5)	18.5	1.00
	Mid-upper	6.4 (0.5)	43.8	5.5 (0.6)	44.4	0.86
	Mid-lower	4.9 (0.7)	33.6	3.6 (0.9)	29.0	0.73
	Basal	1.0 (0.4)	6.8	1.0 (0.4)	8.1	1.00
		14.6 (2.1)		12.4 (2.4)		
Roblin	Distal	2.0 (0.5)	13.1	1.1 (0.4)	23.4	0.55
	Mid-upper	5.4 (0.6)	35.3	2.0 (0.8)	42.6	0.37
	Mid-lower	5.9 (0.9)	38.6	1.3 (0.9)	27.7	0.22
	Basal	2.0 (0.7)	13.1	0.3 (0.2)	6.4	0.15
		15.3 (2.7)		4.7 (2.3)		

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CHAPTER 5

DETERMINATION OF OUTCROSSING IN CUTLER AND ROBLIN WHEAT CULTIVARS BY RANDOMLY AMPLIFIED POLYMORPHIC DNAs (RAPDs)

5.1. INTRODUCTION

The occurrence of offtypes in many semidwarf wheat (*Triticum aestivum* L.) cultivars has raised concern among wheat breeders and controllers of seed quality. One possible cause of offtypes is outcrossing. Since wheat is generally considered a self-pollinating crop, outcrossing has received little attention. In this study, outcrossing was examined in plants of Cutler and Roblin spring wheat cultivars that were moisture stressed and exposed to pollen from the line P8901. Cutler is a semidwarf cultivar that belongs to the Canada Prairie Spring (CPS) wheat class while Roblin is a tall cultivar belonging to the Canada Western Red Spring (CWRS) class. P8901 is a tall, awnless, black-chaff marker line obtained from Dr. R. M. De Pauw (Agriculture and Agri-Food Canada, Swift-Current, Saskatchewan).

The study of outcrossing in crop cultivars requires the availability of characteristic markers that allow for the identification of outcrosses in F_1 or subsequent generations. The methods used to characterize cultivars in wheat can be applied to the identification of outcrosses. The most commonly used methods include phenotypic identification based on morphological traits, and electrophoresis of various proteins (Cooke, 1984; Wrigley, et al, 1982; Ng, et al, 1988). Phenotypic identifications are seldom definitive descriptors, and have a limited use for cultivar identification (Stegemann, 1984). In addition, these methods involve lengthy assessments of plant growth that are costly, labour intensive and subject to environmentally induced variations (Cooke, 1984; Hu and Quiros, 1991).

The laboratory techniques that have been used to characterize wheat cultivar include electrophoresis of isozymes and seed storage proteins (Bassiri, 1976; Arus, et al, 1982; Stegemann, 1984; Ng, et al, 1984). Electrophoresis of seed storage proteins, particularly the gliadins, has been found to be very useful for cultivar identification (Wrigley et al, 1982). The advantage of seed protein electrophoresis techniques over phenotypic assessments is that they are faster, cheaper, and the results are not usually affected by the environmental conditions. However, the protein electrophoresis methods have a drawback in that their ability to detect polymorphisms among closely related genotypes is limited (Hu and Quiros, 1991).

Recent advances in molecular biology have led to the development of techniques that enable the characterization of genotypes based on polymorphic sequences in their DNA. Two types of molecular markers, restriction fragment length polymorphisms (RFLPs), and random amplified polymorphic DNAs (RAPDs) have been used in the development of detailed genetic maps (Michelmore, et al, 1991). Although they can disclose unlimited polymorphic markers, RFLP assays, which involve endonuclease digestions coupled with DNA blot hybridizations, are generally lengthy, and labour intensive (Tingey and del Tufo, 1993).

The RAPD technique developed by Williams et al (1990) and Welsh and McClelland (1990), detects nucleotide sequence polymorphisms based on the amplifications of random DNA sequences by the polymerase chain reaction (PCR), using single primers with arbitrary nucleotide sequences. It is relatively simple to carry out, rapid, has the ability to detect extensive polymorphisms, and requires small amounts of DNA (Hu and Quiros, 1991). RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region. They are usually noted by the presence or absence of an amplification product (Williams et al, 1990; Parks et al, 1991; Rafalski et al, 1994). The presence of each amplification product implies complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotide primer at each end of the amplified product (Tingey and del Tufo, 1993). Each primer will direct the amplification of discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphisms between individuals. RAPDs are dominant markers that segregate in Mendelian fashion. They have been used effectively as markers for specific genes during the development of near isogenic lines, and in selection for disease resistance (Williams et al, 1990; Michelmore et al, 1991; Klein-Lankhorst et al, 1991; Martin et al, 1991).

In this study, outcrossing in Cutler and Roblin was determined by studying the RAPD marker polymorphisms of their F_1 progeny. Results from previous determinations of outcrossing in Cutler and Roblin, using both phenotypic characters and electrophoresis of seed storage proteins, suggested that moisture-stressed Cutler has a higher potential for outcrossing compared with moisture stressed Roblin.

5.2. MATERIALS AND METHODS

5.2.1. PLANT MATERIAL

Seed of Cutler, Roblin, P8901, F_1 seed of Cutler x P8901 and Roblin x P8901 crosses, and F_1 seed from plants of Cutler and Roblin that were moisture stressed and exposed to pollen from P8901, were planted in 4-inch diameter, 4-inch deep pots in the greenhouse. The spikes from which the seed was harvested had been divided into four approximately equal regions designated as the basal region, mid-lower, mid-upper and distal regions. Each pot was labelled to indicate the region of spike the seed came from. One seed was planted per pot using a medium containing coarse sand, peat moss and vermiculite in the ratio of 1: 2: 2 by volume. The temperature and photoperiod in the greenhouse were set at 22°C/18°C and 16h/8h day/night, respectively. The lighting was supplemented using 400W sodium lamps to give a light intensity of about 450 $\mu E\ m^{-2}s^{-1}$ at the pot level. The pots were watered daily until the plants were 4 weeks old, then they were transferred to a dark room for two days (to stop photosynthesis and reduce the concentration of starch in the tissues) before harvesting the shoots. For Cutler, Roblin, P8901, Cutler x P8901 and Roblin x P8901, shoots were harvested from several plants and bulked to give about 5g (fresh weight) samples. Samples for the F_1 plants of Cutler and Roblin, whose parents were exposed to pollen from P8901, were obtained from single plants. The samples were frozen in liquid nitrogen immediately after harvesting, freeze dried, and stored in a desiccator at -20°C. The pots that shoots were harvested from were returned to the greenhouse, where tillers grew from the lower nodes of most of the plants. Phenotypic observations were made on the tillers after they headed.

5.2.2. DNA EXTRACTION

Freeze-dried samples were ground to a fine powder and transferred into a mortar where 10 ml of pre-heated (60°C) hexadecyl-trimethyl ammonium bromide (CTAB) containing 1.5µl 2-mercaptoethanol was added. Additional grinding was done and the paste was transferred to a 50 ml Oakridge tube, where 1 ml 20% SDS and 100 µl proteinase K were added, and incubated for 1h in a 55°C water bath. 5 ml 5M potassium acetate was added and cooled in ice for 20 min. The samples were centrifuged at 25,000 x g for 10 min and an equal volume of chloroform/isoamyl alcohol (24:1) was added to the supernatant, mixed, and centrifuged for 5 min at 3500 rpm in a clinical centrifuge. DNA was precipitated from the aqueous (upper layer) phase by adding 0.6 vol. isopropanol. The DNA pellet was transferred to a 1.5 ml centrifuge tube, washed with a buffer (70% ethanol, 10 mM sodium acetate), dried in a Speed Vac, and re-suspended in 500 µl RNase buffer (10 mM Tris pH 7, 15 mM NaCl). 5 µl RNase A (5 µg/µl) was added and incubated for 1 h in a 37°C water-bath.

An equal volume of phenol:chloroform mixture (250 µl each) was added, mixed, and the layers separated by centrifuging at 13,000 rpm in a microcentrifuge. The upper phase was washed two times with an equal volume of chloroform/ isoamyl alcohol, then an equal volume of 4 M NaCl was added and allowed to stand for 10 min. DNA was precipitated by adding 100 µl 7.5 M ammonium acetate and 750 µl 95 % ethanol. The pellet was then rinsed three times in 70 % ethanol, dried in a Speed Vac, and re-suspended in 400 µl TE buffer (10 mM Tris, 1 mM EDTA pH 8). The concentration of DNA in each sample was determined using a U.V. spectrophotometer, and samples with DNA concentration of 5 ng/µl were prepared. All samples were stored at -20°C.

5.2.3. RANDOMLY AMPLIFIED POLYMORPHIC DNA ANALYSIS

PCR reaction mixtures containing 15.3 µl water, 1.9 µl 10 mM $MgCl_2$, 2.5 µl 10X PCR buffer, 0.5 µl 10 mM dNTP, 0.2 µl Taq DNA polymerase, 2.5 µl of 2 µM primer and 2 µl of DNA sample containing 5 ng/µl were prepared. A single 10-base, random primer from either the University of British Columbia (UBC) or Operon Technologies (OPA) was used in each reaction. Each reaction mixture was overlayed with a drop of oil and 45 cycles of PCR were carried out using denaturation temperature of 94°C for 1 min, annealing temperature of 34°C for 1 min, and polymerization temperature of 72°C for 2 min. An additional cycle in which the polymerization step was extended to 5 min was included. The PCR products were separated by electrophoresis in a 1.4% agarose gel containing 1:20,000 v/v ethidium bromide. The gels were visualized under u.v light, and photographed using type 57 black and white instant polaroid film. To enable the determination of band sizes, a 1 kb DNA ladder was loaded in one lane of each gel. In all, 90 10-base primers from the University of British Columbia (UBC301-UBC390), and 20 primers from Operon technologies (OPA1- OPA20) were screened to obtain the primers that could be used to determine outcrossing in Cutler and Roblin. The primers that resulted in polymorphic bands between Cutler or Roblin and P8901, and enabled the detection of the polymorphic bands of P8901 in the F_1 crosses were used in subsequent PCR reactions to determine the outcrosses among the F_1 plants of Cutler and Roblin whose parents had been exposed to pollen from P8901.

5.3 RESULTS AND DISCUSSION

Primers that resulted in polymorphic bands that could be used to determine outcrossing in both Cutler and Roblin were uncommon. Several primers failed to show polymorphisms. Some primers failed to amplify DNA from either parent, while others amplified DNA from only one parent.

Out of the 110 primers screened, only primer OPA 16 (sequence= $^{5'}$AGCCAGCGAA$^{3'}$) from Operon Technologies amplified a polymorphic sequence in P8901 that could be used to identify outcrosses in Cutler. PCR reactions using this primer amplified a 700 kb band that was present in P8901 and in the Cutler X P8901 cross, but absent in Cutler (Figures 5.1a and 5.1b). Similarly, there was only one primer, UBC 324 (sequence= $^{5'}$ACAGGGAACG$^{3'}$), that amplified products that could be used to identify outcrosses in Roblin. This primer resulted in the amplification of three polymorphic bands (550 kb, 750 kb and 1200 kb), which were present in P8901 and in the Roblin X P8901 cross but absent in Roblin (Figure 5.2a and 5.2b).

Outcrossing was determined in Cutler and Roblin by running PCR reactions on DNA samples of their progeny using primers OPA 16 and UBC 324, respectively. Out of 33 F_1 plants of Cutler, 26 plants showed the 700 kb polymorphic band, indicating outcrossing of about 79%. In Roblin, 27 of 40 F_1 plants tested had all or some of the polymorphic bands of P8901 (550 kb, 750 kb, 1200 kb), indicating an outcrossing of about 67%. These results support those obtained from using phenotypic markers (Chapter

3) and seed protein electrophoregram (Chapter 4), which indicated that Cutler has a higher potential for outcrossing compared with Roblin.

There was a smaller difference between the levels of outcrossing in Cutler and Roblin in this experiment compared with the differences observed when phenotype or seed protein electrophoregrams were used. When outcrossing was determined using the phenotype of the F_1 plants, moisture-stressed Cutler and Roblin showed outcrossing levels of 78 and 52%, respectively. Using electrophoresis of gliadin proteins, outcrossing levels of 84% in Cutler and 37% in Roblin were detected. These differences could be caused partly by the differences in the number of seeds/plants tested and partly by differences in the accuracy of the different methods used to identify the outcrosses. The phenotypic test, for example, could have under estimated outcrossing in Roblin if there were any outcrosses that failed to express the black chaff colour since there were no other markers. Gliadin electrophoresis produces a limited number of polymorphic bands. The intensities of the polymorphic bands in an F_1 cross are usually lower than those in the parents, and may at times be difficult to identify outcrosses. The RAPD technique enables the identification of outcrosses to be made with much more accuracy since it is possible to generate a large number of polymorphisms by changing the primers. Unlike protein electrophoregrams, RAPDs can detect differences in both coding and non coding regions of the gene, including sequence differences that may exist between genes coding for the same protein. Also RAPDs are usually dominant markers (Williams et al, 1990) and the intensity of the polymorphic bands in an F_1 cross is similar to that observed in the parents hence it is easier to detect outcrosses.

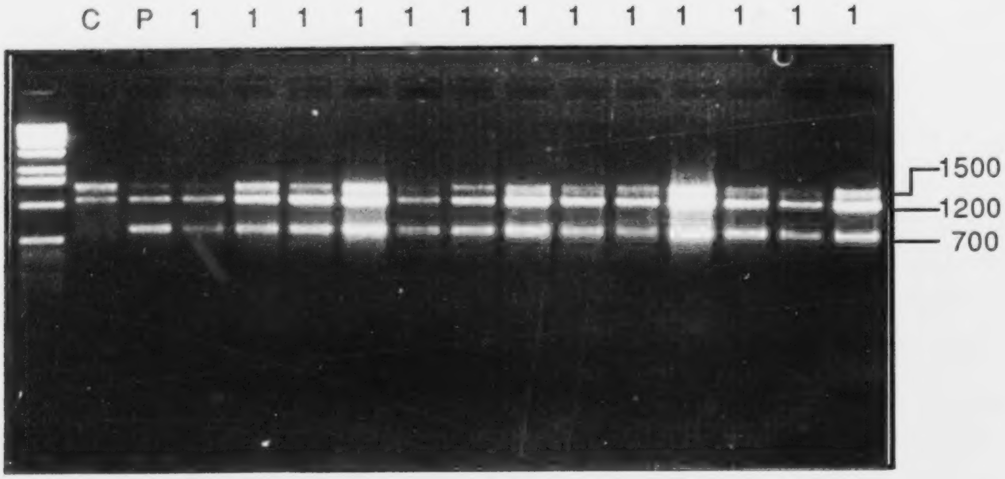
Seed distribution in the spikes of Cutler and Roblin from which the F_1 seed was obtained was similar to that observed in the protein electrophoresis experiment (Chapter 4). Cutler had the highest seed set in mid-upper region followed by the mid-lower, distal, and the basal regions (Figure 5.3a). Also the distribution of outcrosses in Cutler was similar to those obtained using phenotypic markers or seed protein electrophoregrams, with the highest proportion of outcrosses occurring in the mid-upper and mid-lower regions of the spike (Figure 5.3b; Table 5.1). In Roblin, the mid-lower region, which was found to have a low frequency of outcrosses in previous studies (Chapters 3 and 4) showed a relatively high frequency of outcrosses. 33.3% of the outcrosses in Roblin were found in the mid-lower region while the distal region had only 18.5%. The wide variation in the extent of outcrossing in spikes of Roblin, however, led to high standard errors (Figure 5.3b; Table 5.1), hence comparison of outcrossing among the regions could not be made. The outcrossing indices for the different regions of spike in Cutler showed different patterns compared with those obtained from seed protein or phenotypic markers. It had the lowest outcrossing index in the distal region and the highest in the basal region (Table 5.1). Roblin had the highest outcrossing index in the basal region but, like in the previous experiments, it still had the lowest outcrossing index in the mid-lower region.

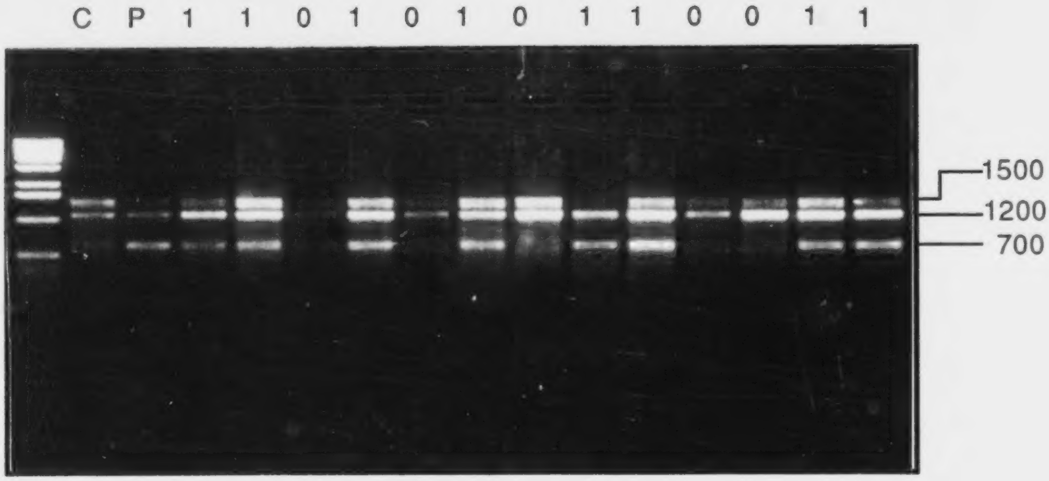
Most of the tillers that came from the lower nodes of Cutler and Roblin after the shoots were harvested for DNA extraction grew to the heading stage. From the tillers, outcrosses could not be determined using height, maturity or expression of the black chaff colour. However, it was possible to determine outcrossing in Cutler based on awnedness. Of 32 plants of Cutler whose tillers grew to the heading stage, 26 were awnless while 6 were awned. RAPDs analysis identified 25 of the 26 awnless plants to be outcrosses since they all showed the 700 kb band characteristic to P8901.The close agreement in the two results suggest that the two methods can effectively be used to determine outcrossing in wheat. The single F_1 plant that was awnless but did not express the 700 kb band was possibly the result of a cross with a different awnless cultivar.

CONCLUSIONS

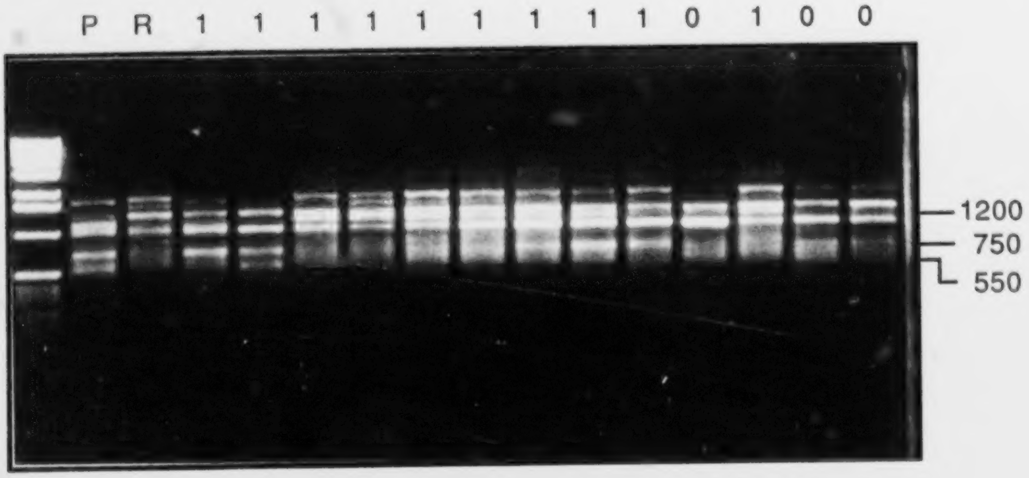
Although several random 10-base primers amplified DNA sequences from Cutler, Roblin and P8901, only two primers amplified polymorphic sequences that could be used to study outcrossing in Cutler and Roblin. Cutler showed a higher frequency of outcrosses compared with Roblin, suggesting that under moisture-stress conditions, Cutler has a higher potential for outcrossing compared with Roblin. Differences in the frequency of outcrossing in the different regions of the spike could not be observed, as a result of large standard errors particularly in Roblin. The fact that all the Cutler F_1 plants that showed the polymorphic band characteristic to P8901 were also awnless indicates that the RAPD technique can reliably be used to study outcrossing in wheat.

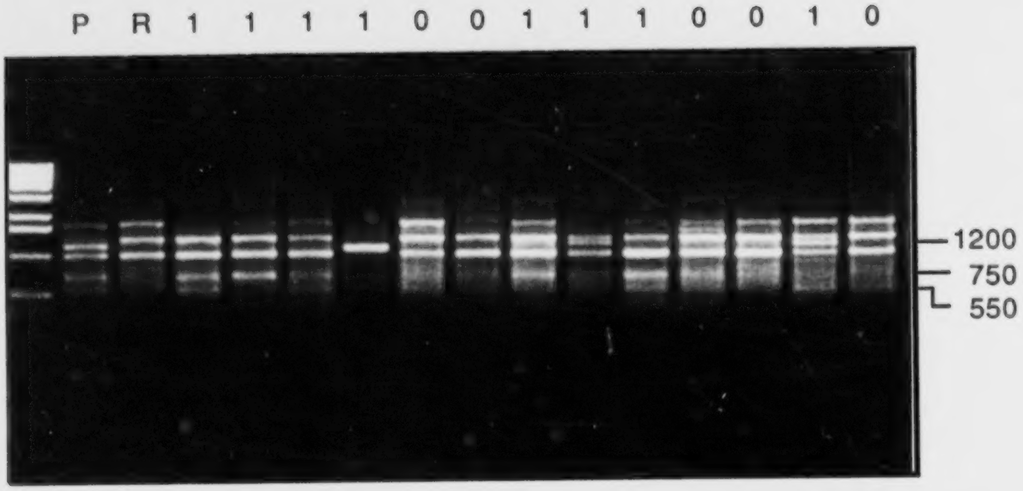
Figures 5.1a and 5.1b. Electrophoregrams of amplified products from DNA of Cutler (C), P8901 (P), and F_1 plants of Cutler exposed to pollen from P8901, following PCR using primer OPA 16. Lanes of F_1 plants are labelled '1' (outcrossed) or '0' (non-outcrossed), depending on whether or not they exhibit the 700kb band. 1kb DNA ladder is on the left in each gel.


C P 1 1 1 1 1 1 1 1 1 1 1 1 1
1500
1200
700


C P 1 1 0 1 0 1 0 1 1 0 0 1 1
1500
1200
700

Figures 5.2a and 5.2b. Electrophoregrams of amplified products from the DNA of P8901 (P), Roblin (R), and F_1 plants of Roblin exposed to pollen from P8901, following PCR using primer UBC 324. Lanes of F_1 plants are labelled '1' (outcrossed) or '0' (non-outcrossed), depending on whether or not they exhibit the polymorphic bands of P8901. 1kb DNA ladder is on the left in each gel.


P R 1 1 1 1 1 1 1 1 1 0 1 0 0
1200
750
550


P R 1 1 1 1 0 0 1 1 1 0 0 1 0
1200
750
550

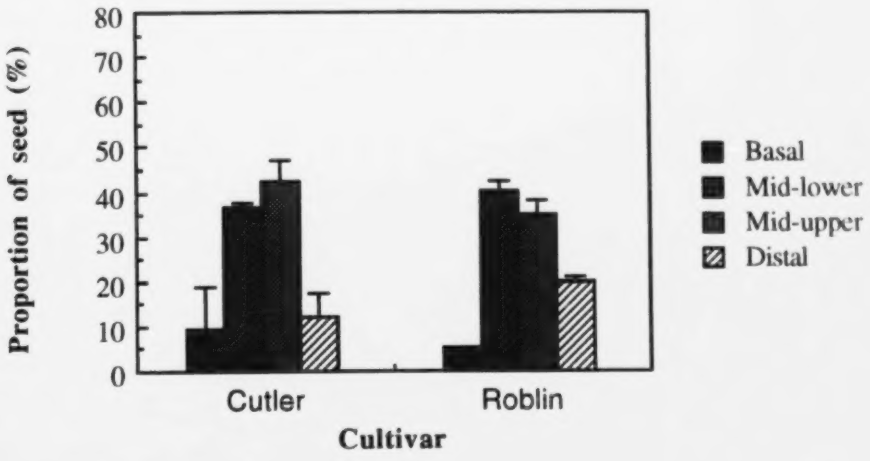


Figure 5.3a. Seed distribution across the spike in plants of Cutler and Roblin that were moisture stressed and exposed to pollen from P8901 at flowering. The values represent the number of seeds per region expressed as a percentage of the number of seeds per spike. (Mean ± SE, n=2).

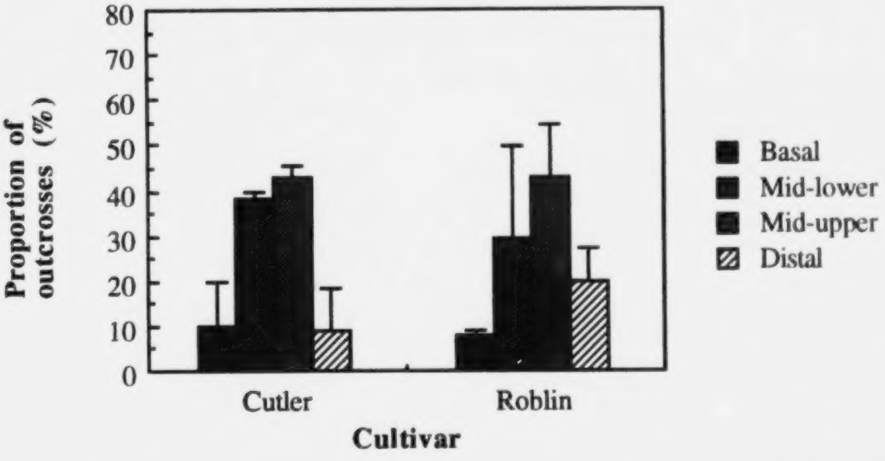


Figure 5.3b. Distribution of outcrossed seed across the spike in plants of Cutler and Roblin that were moisture stressed and exposed to pollen from P8901 at flowering. The values represent the number of outcrossed seeds per region expressed as a percentage of the number of outcrossed seeds per spike. (Mean ± SE, n=2).

Table 5.1. Frequency of outcrosses per region of spike in moisture stressed plants of Cutler and Roblin exposed to pollen from P8901. Outcrosses were determined based on expression of the polymorphic RAPD markers of P8901 in the F_1 plants. The outcrossing index is the ratio of the number of outcrossed seeds found in a region to the number of seeds found in that region. Standard errors are shown in parentheses.

CULTIVAR	Region	Number of seeds per region [A]	Proportion of seed per region (%)	Number of outcrosses per region [B]	Proportion of outcrosses per region (%)	Outcross-ing index [B]/[A]
Cutler	Distal	2.0 (1.0)	12.1	1.0 (1.0)	7.7	0.50
	Mid-upper	7.0 (1.0)	42.4	5.5 (0.5)	42.3	0.76
	Mid-lower	6.0 (0.0)	36.4	5.0 (1.0)	38.5	0.83
	Basal	1.5 (1.5)	9.1	1.5 (1.5)	11.5	1.00
		16.5 (3.5)		13.0 (4.0)		
Roblin	Distal	4.0 (0.0)	20.0	2.5 (0.5)	18.5	0.63
	Mid-upper	7.0 (1.0)	35.0	5.5 (0.5)	40.7	0.79
	Mid-lower	8.0 (0.0)	40.0	4.5 (3.5)	33.3	0.56
	Basal	1.0 (0.0)	5.0	1.0 (0.0)	7.4	1.00
		20.0 (1.0)		13.5 (4.5)		

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CHAPTER 6

SUMMARY AND CONCLUSIONS

The introduction of semidwarf wheat has resulted in considerable increases in wheat yields worldwide (Lupton, 1987). Apart from imparting lodging resistance, the dwarfing genes have also improved other yield parameters such as harvest index (Jain and Kulshrestha, 1976). Semidwarf cultivars have been reported to out-yield tall cultivars under favourable moisture conditions, but are more susceptible to drought and high temperature stress (Syme, 1969; McNeal et al, 1972; Laing and Fisher, 1977).

Most of the spring wheat grown in western Canada is of the Hard Red Spring type, and is classified under the Canada Western Red Spring (CWRS) wheat class. This class has mainly tall cultivars with high protein content and excellent baking and milling quality (Kibite, 1986). Wheat cultivars in this class, however, are relatively low yielding. Semidwarf cultivars were developed for the Western Canada region to improve wheat yields (Briggs, 1985; Wolfe and Clarke, 1985; Kibite, 1986). The Canada Prairie Spring (CPS) wheat class is composed mainly of semidwarf cultivars, characterized by high yields and good milling quality, but with lower protein content and inferior baking quality compared with CWRS cultivars. The CPS cultivars have been reported to out-yield CWRS cultivars by between 25 and 35% (Wolfe and Clarke, 1985).

Many cases of offtype plants have been reported in semidwarf wheat, including most of the CPS cultivars so far released (Worland and Law, 1985; Storlie and Talbert, 1993; Briggs, pers. comm.). The possible causes of offtypes in wheat include accidental mixing of seed, occurrence of volunteer plants in the field, genetic abnormalities and outcrossing. Offtypes found in the CPS cultivar Cutler reoccurred after rogueing (Briggs, pers. comm.), and their progeny segregated for height and awnedness, suggesting that they did not arise from seed mixing or volunteer plants. Also, seed protein electrophoregrams of these offtypes were different from Cutler's, suggesting that they were not aneuploids. These observations indicate that the offtypes found in Cutler, and possibly those in other CPS cultivars, are probably a result of outcrossing.

Wheat florets are generally self pollinated since they are enclosed by glumes, and open only briefly at anthesis (Leighty and Sando, 1924). Natural outcrossing in cultivated wheat varieties has been reported to range from 0-4% (Heyne and Smith, 1967). However, certain environmental factors, particularly those that induce male sterility may lead to increased levels of outcrossing in wheat. Floret sterility caused by pre-anthesis moisture stress has been reported to result mainly from pollen infertility (Bingham, 1966; Morgan, 1980; Saini and Aspinall, 1981). Male sterile florets were found to open wider and for longer periods than fertile florets (Saran et al, 1971). This would increase the potential for outcrossing in the presence of an external pollen mass.

In a greenhouse experiment to determine the effect of moisture stress on floret sterility in five CPS cultivars (Biggar, Taber, Cutler, CDC 1 and Oslo), and three CWRS cultivars (Roblin, Park and Katepwa), it was found that the frequency of sterile florets in both CPS and CWRS cultivars increased with moisture stress. However, sterility in CPS cultivars increased more than in the CWRS cultivars when the plants were exposed to increasing levels of stress. At the highest level of moisture stress (watering at 7-day intervals), all the semidwarf cultivars had significantly higher ($p=0.01$) levels of sterility compared with the CWRS cultivars.

The rate of moisture loss from pots of Biggar was higher than that observed in the other cultivars. Pots of CPS cultivars on average lost moisture at a higher rate compared with those of CWRS cultivars, although the difference was not significant. Tolerance to moisture stress was estimated in Cutler, CDC 1, Katepwa and Roblin by measuring the electrolyte leakage that occurred in leaf samples that were desiccated in polyethylene glycol (PEG). Leaves of well-watered Cutler showed the greatest injury (25%) following incubation in 30% PEG while Roblin showed the least injury (0.5%). This suggest that

Cutler is the most susceptible to moisture stress, and that Roblin is inherently tolerant. Leaves from moisture-stressed plants of Cutler and Roblin, however, behaved differently with Cutler showing no injury and Roblin showing an injury of 21% after incubation in 30% PEG. This suggests that Cutler has a greater ability to adjust to moisture stress compared with Roblin.

Stigma receptivity and pollen fertility studies were conducted to determine the cause of floret sterility in moisture-stressed wheat. Moisture stress reduced pollen fertility in the four cultivars that were tested (Cutler, CDC1, Katepwa and Roblin). Katepwa showed the greatest reduction in pollen fertility while the least reduction occurred in Roblin. Stigma receptivity was examined by studying the seed set in Cutler and Roblin. Moisture stress significantly ($p=0.05$) reduced seed set in both cultivars, with Cutler showing a greater reduction than Roblin. When florets of moisture-stressed plants of Cutler and Roblin were hand pollinated, seed set was increased from 21.5 to 62% in Cutler and from 59.8 to 72% in Roblin, indicating that there were some sterile florets in the two cultivars that had receptive stigmas. The fact that there was a significant increase in seed set in moisture-stressed Cutler following hand pollination indicates that most of the floret sterility resulted from lack of viable pollen.

Moisture-stressed plants of Cutler and Roblin, apart from showing increased frequencies of male sterile florets, also showed a wider opening of florets compared with well-watered plants. This further increased the chances of outcrossing. The potential for outcrossing in moisture stressed plants of Cutler and Roblin was investigated by exposing the plants to pollen from a marker line (P8901), then observing changes in seed set, and determining the number of outcrossed seeds in each spike. Exposure of moisture-stressed plants of Cutler and Roblin to pollen from P8901 resulted in a significant increase in seed set in Cutler while in Roblin there was a small, non significant increase in seed set. Assuming that the increase in seed set was a result of the added pollen, these results suggest that moisture stressed Cutler has a higher potential for outcrossing compared with moisture stressed Roblin.

Three methods were used to determine the level of outcrossing outcrossing. One was a progeny test in which seeds from spikes exposed to pollen from P8901 were planted and phenotypic observation made on the F_1 plants to determine those that expressed P8901 characteristics, including the black chaff colour. In the second method, gliadin electrophoregrams were used to determine the number of seeds that expressed the polymorphic bands of P8901. Thirdly, outcrossing was determined by running PCR reactions on the DNA from the F_1 plants using random primers, and identifying the plants that expressed the RAPD markers of P8901.

Results from all three methods indicated that there was more outcrossing in Cutler than in Roblin. The difference in the levels of outcrossing observed in moisture-stressed Cutler and Roblin, however, varied depending on the method of determination (Table 6.1). Using the gliadin electrophoresis method, for example, outcrossing levels of 84% in Cutler and 37% in Roblin were observed while the outcrossing levels determined using the RAPD method were 79% (Cutler) and 67% (Roblin). Part of this variation may be a result of differences in the composition of samples tested in each experiment. Although the plants received similar treatments, outcrossing levels, particularly in Roblin, varied widely between plants with a range of < 10% in some plants to > 80% in others.

The accuracy of the methods used is another possible source of variation. In the phenotypic test, for example, the presence of some tall, awnless plants that did not clearly express the black chaff colour suggests that some outcrosses may not be detected using the phenotypic test alone. The limitations of the gliadin protein electrophoresis method during cultivar identification include the inability to distinguish between closely related genotypes, as a result of limited polymorphisms. Also, the polymorphic bands in an F_1 seed usually appear weaker than in the parents and occasionally may be difficult to observe. Such cases could result in errors in the determination of outcrossing. In this study some of the bands that were polymorphic between the parents (Cutler or Roblin, and P8901) had strong intensities. Although the intensities of these bands in the F_1 seeds were lower compared with those observed in the parents, they were still strong enough to enable unambiguous detection of outcrosses in most of the seeds tested.

The RAPD technique offers one of the most accurate means of identifying outcrossed seeds.The fact that a large number of polymorphic bands can be generated means that polymorphisms can be detected even between closely related genotypes. Since RAPD markers usually behave as dominant markers, their intensities in the outcrossed plants are similar to those observed in the parents, making it easier to identify the outcrosses. Also, the technique has been shown to be able to detect rare DNA sequences and hence can be used to detect outcrosses in seed bulks.This can be very useful in situations where outcrossing in large seed stocks such as those obtained from field experiments is being studied.

In situations where outcrossing is determined by analyzing single seeds (as was the case in this study) the RAPD technology may be limited by the number of samples that can be analyzed. This mainly arises from the fact that DNA extraction procedure is long and tedious. Part of the difference in the results obtained from the gliadin electrophoresis and those obtained from the RAPD experiment could be a result of sample size since about twice as many samples were analyzed in the gliadin electrophoresis experiment.

From this study it can be concluded that under moisture stress conditions, CPS cultivars have a higher frequency of sterile florets compared with CWRS cultivars. Most of the floret sterility observed in the CPS cultivar Cutler was a result of male sterility, since a large increase in seed set was observed following hand pollination. The frequency of outcrosses was higher in Cutler compared with Roblin in all the experiments, indicating that moisture-stressed Cutler has a higher potential for outcrossing compared with moisture-stressed Roblin.

Table 6.1. Outcrossing levels in Cutler and Roblin wheat cultivars determined by examination of phenotypic characters, electrophoresis of gliadin proteins, and RAPDs analysis. Plants were moisture stressed and exposed to pollen from P8901 at flowering.

CULTIVAR	Outcrossing based on phenotypic characters (%)	Outcrossing based on gliadin electrophoregrams (%)	Outcrossing based on RAPDs (%)
Cutler	78	84	79
Roblin	52	37	67

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